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In order to determine whether differences exist in expression of estrogen receptor (ER) variant mRNAs between normal and tumor breast tissues, we have analyzed by reverse transcription-polymerase chain reaction (RT-PCR) ER variants mRNA expression in matched normal breast tissue and breast tumor components from 18 different patients. A higher expression of exon 5-deleted, and of clone 4 ER variants in the tumor component compared to the normal counterpart of matched samples was seen. A general trend toward a higher expression of exon 3-deleted ER variant in the normal tissue was also observed. This is consistent with previous observations made on independent samples. We have described the presence within normal breast as well as in breast tumor tissues of several variant forms of ER–β mRNA deleted in exon 5, exon 6 and in exon 5+6 sequences. The biological significance of the presence of these variants and of wild-type ER–β in breast tissue, in particular their role in estrogen/antiestrogen action, remains to be determined. We showed that a significantly ($p<0.02$) higher ER–α/ER–β ratio was observed in the breast tumors compared with their matched normal breats tissues and that this increase was attributed to a significant ($p<0.05$) increase in ER–α mRNA expression and a lower ER–β mRNA expression in the tumor compared with that of the normal component in some ER+ cases. Our results suggest that the role of ER–α and ER–β driven pathways and/or their interaction change during breast tumorigenesis.

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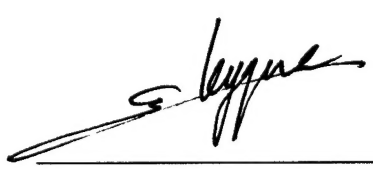

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TABLE OF CONTENTS

INTRODUCTION

Estrogen is known to be an important regulator of normal breast tissue growth and tumor development.[1] When this proposal was submitted, estrogens were thought to act mainly through a single receptor, now referred to as ER–α. ER–α is a ligand–activated transcription factor that belongs to the steroid/retinoic acid/thyroid receptor super family.[2] ER–α mRNA contains 8 different exons encoding a protein divided into structural and functional domains (A-F) (see Figure 1).[3] Region A/B of the receptor is implicated in trans-activating function 1 (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another trans-activating function (AF-2).

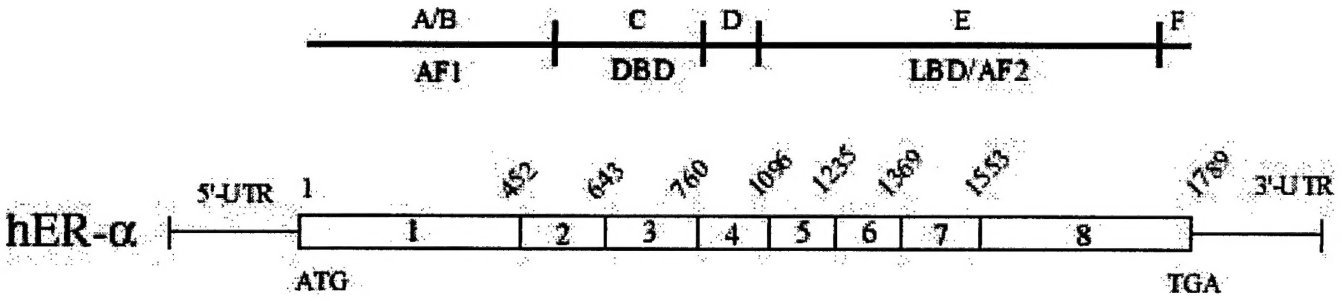

Figure 1: Schematic representation of ER–α cDNA and of the functional and structural domain of hER–α

Several ER–α variant mRNAs have been described, that are missing one or more of the exons contained in the wild-type (WT). For a review of the different ER–α variants and their putative functions, see Appendix 1.[4] The putative encoded proteins lack some of the WT-ER–α structural domains. Some of these proteins, such those encoded by exon 5-deleted and exon-3- deleted ER–α variant mRNAs have been shown to interfer with wild-type ER–α signal transduction. ER–α variants have been suspected to be involved in the acquisition of estrogen-independence that occurs during breast tumor progression.

This hypothesis is supported by the observation that exon 5-deleted ER–α mRNA expression relative to WT-ER–α is higher in ER-/PR+ than in ER+/PR+ tumors[5]. More recently, Gallachi et al. reported that an increased expression of exon 5-deleted ER–α variant mRNA levels was observed in tumors relapsing within 15 months compared to tumors not relapsing within the same amount of time.[6] Exon 7-deleted mRNA variant

expression was also shown to be higher in ER+/PR- than in ER+/PR+ tumors.[7] Similarly, relatively higher levels of the clone 4 truncated variant ER–α mRNA were found in tumors with markers of poor prognosis and lack of hormone sensitivity compared to those with markers of good prognosis and hormone sensitivity.[8] We have recently demonstrated that expression of exon 5-deleted variant mRNA relative to WT-ER–α, was higher in breast cancer than in normal breast tissue.[9] Similarly, we have established that clone 4 mRNA expression relative to WT was significantly increased in a group of breast tumors (all ER+/PR+) compared to unmatched normal reduction mammoplasty samples.[10] Such data suggest that the molecular mechanisms generating ER variant mRNAs could be deregulated in breast cancer tissues compared to normal breast tissues, and may contribute to early steps in breast tumorigenesis.

The goal of this project is to address the possible role of Estrogen Receptor variants in human breast tumorigenesis.

Objectives:

1. To look for differences in the expression of already described forms of ER–α variant mRNAs between matched normal breast tissue, invasive primary carcinoma, and metastatic carcinoma in axillary lymph nodes.
2. To identify variant ER mRNAs differentially expressed in normal breast and breast cancer tissue.
3. To determine the putative function of differentially expressed variants.

To reduce the possible impact of patient variability, this study made comparisons between matched tissues (normal breast tissue, invasive primary carcinoma, and metastatic carcinoma in axillary lymph nodes) samples coming from the same patient. All tissue specimens were provided by the Manitoba Breast Tumor Bank.

The recent cloning of a new estrogen receptor,[11] now referred to as ER–β, led to a total re-evaluation of estrogen signal transduction in target tissues. This receptor shares the same structural and functional composition as the ER–α and has strong sequence similarities within the DNA binding domain and the ligand binding with ER–α (Figure 2).

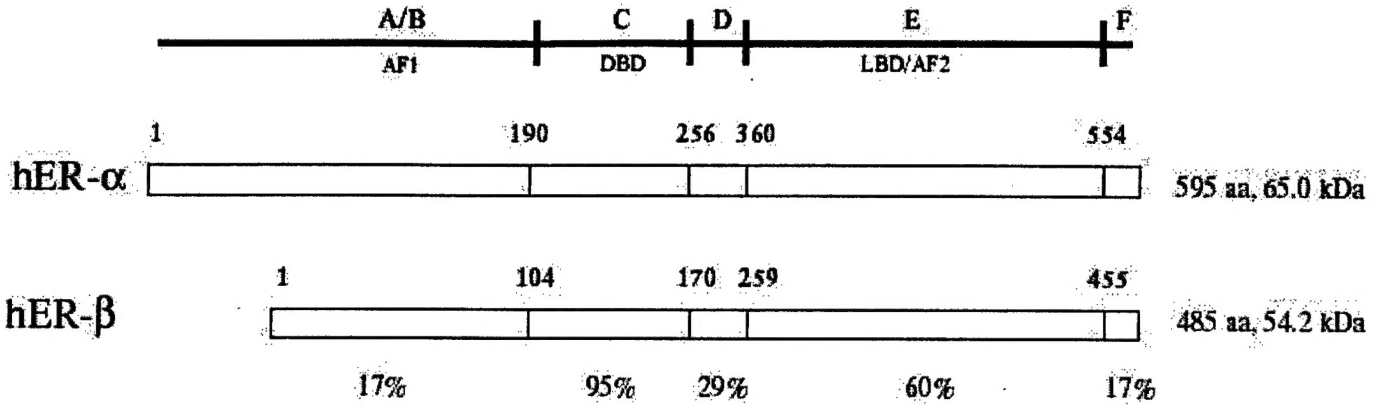

Figure 2: Sequence similarities between ER–α and ER–β

This new receptor is able to form heterodimers with ER–α and data have been accumulating which suggest cross-talk between ER–β and ER–α signalling pathways. For a review of ER–β characteristics, see Appendix 2.[12] It now appears that the action of estrogen has to be considered in the context of the two signaling pathways. We reported last year the detection of ER–β mRNA in some human breast tumor.[13] This observation led us to include additional objectives in our project.

4. Do equivalent ER–β variants exist ?

5. Does the balance between ER–α and ER–β change during tumor progression ?

BODY

Objective 1

Comparison of the relative expression Exon 3-deleted, exon 5-deleted and clone 4 ER variant mRNAs in matched normal or tumorous breast tissues.

In our previous report, we described a general trend toward a higher expression of exon 5-deleted, exon 7-deleted ER variant and clone 4 ER variant in the tumor component compared to the normal counterpart of matched samples. This was consistent with the observations made on independent samples.[9,10] These data,

obtained on 10 cases failed to reach statistical significance. We concluded that the number of cases needed to be increased in order to establish confidently any statistically significant differences. We also noted that the phenotypic characteristics of the tumors (ER and PR values, as measured by ligand binding assay) were parameters that will be taken into account in the selection of additional cases.

Interestingly, a recent report suggested a decrease in the expression of the exon 3-deleted ER–α variant mRNA in breast tumor compared to independent normal breast epithelial cells.[14] We therefore included an analysis of the expression of this variant in the matched normal and tumor samples.

We selected a new set of cases in the Manitoba Tumor Data Bank files. Eighteen patients have been identified from which matched normal breast tissue and primary invasive carcinoma were available. Among these cases, 6 are ER-/PR- (i.e ER levels lower than 3 fmol/mg protein and PR levels lower than 10 fmol/mg, as determined by ligand binding assay), 1 is ER-/PR+, 2 are ER+/PR-, and 9 are ER+/PR+.

Detection, analysis and quantitation of exon 5-deleted and exon 3-deleted mRNAs was performed by RT-PCR as previously described.[9] Briefly, for each patient, total RNA was extracted from the normal and tumor components of 20 μm frozen cryostat sections. Reverse transcription of total RNA using random hexamers was followed by PCR amplification using appropriate primer sets and dCTP [α-^{32}P]. PCR products were separated on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with an intensifying screen.

For the quantification of clone 4 variant mRNA, and as underlined in our previous report, we have redesigned our triple-primer polymerase chain reaction (TP-PCR) assay that we had previously set up to quantify clone 4 variant ER mRNA expression. [10] This new TP-PCR, that uses new primers, has been validated by comparing the results obtained using that approach to those obtained using a standardized RNase protection assay. These data are in press in the British Journal of Cancer appended (see appendix 1).[15] We showed that this approach is reliable and highly specific, and can be used to address the question of the expression of clone 4 variant mRNA relative expression in ER negative samples or samples having a very low ER, by binding assay. Clone 4 expression has therefore been assayed on the selected subset of 18 patients previously described and in

which exon 5- and exon 3-deleted ER–α variant expression was measured. Briefly, for each patient, total RNA was extracted from the normal and tumor components of 20 µm frozen cryostat sections. Reverse transcription of total RNA using random hexamers was followed by PCR amplification using three primers and dCTP [α-^{32}P]. PCR products were separated on 6% polyacrylamide gels and following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with an intensifying screen.

For each analysis, quantification of signals was carried out after excision of the bands corresponding to variant and WT mRNA (using autoradiographs as a guide), followed by addition of 5 ml scintillant (ICN Pharmaceuticals, Inc, Irvine, California) and counting in a scintillation counter (Beckman). The ratio between variant signal and WT-ER signal was calculated. For each experiment, the ratio observed in the same particular tumor (case number 12), was arbitrarily attributed the value of one and all other ratios expressed relatively. This normalization eliminates any possible variation resulting from different inter-experimental parameters, such as batches of label or Taq polymerase etc...

For each sample, at least three independent PCR assays were performed. Statistical analysis of the comparison between ratios observed in normal and matched tumor compartment was by the Wilcoxon's matched pair test.

Results obtained are summarized in figure 3 (exon 5-deleted ER variant), figure 4 (exon 3-deleted ER variant) and Figure 5 (clone 4 variant).

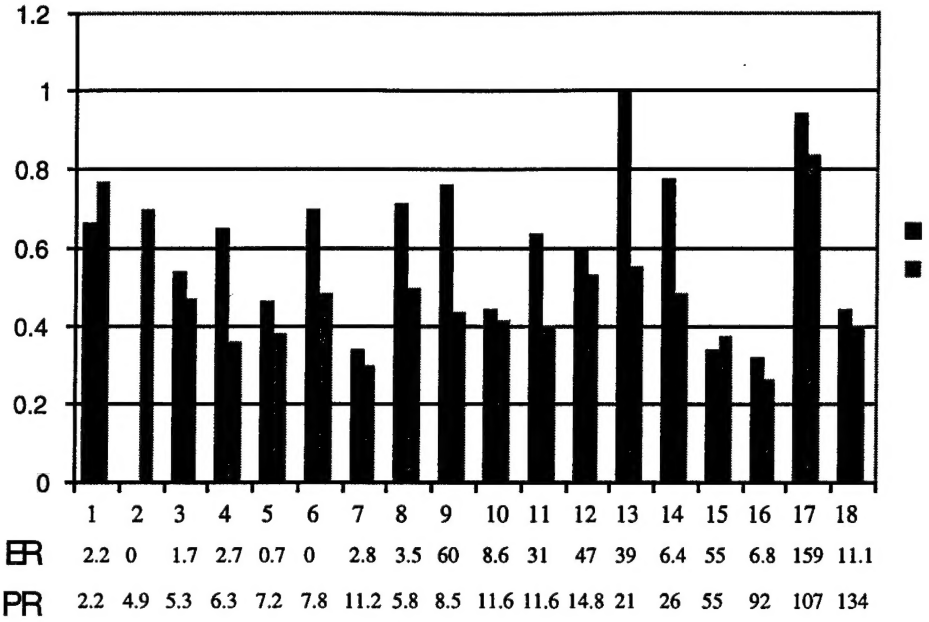

Figure 3: For each patient (1-18) normalized exon 5-deleted signal/WT-ER signal ratio (D5) is indicated for both normal (gray) and primary invasive lesion (black). ER and PR levels of the tumor, as determined by ligand binding assay, are also indicated.

One of the patients (patient number 2) did not express detectable ER mRNA within its tumor compartment using this primer set and was therefore withdrawn from the statistical cohort analyzed. The absence of signal in this particular tumor component is likely to result from its very low ER–α content, also suggested by the value (O) observed by ligand binding assay. A lower exon 5-deleted expression in normal than in the matched tumor component is observed in 15 out of 17 patients. Considering the total cohort, the difference observed reached statistical significance ($n=17$, $p<0.01$). Similarly this difference between matched normal and tumor component is also statistically significant when considering ER+ ($n=11$, $p<0.003$) and ER+/PR+ ($n=9$, $p<0.02$) subgroups only. A similar trend towards an higher exon 5-deleted variant mRNA in tumor compartment (5 out of 6 patients) is observed in ER- subgroup even though not significant, probably because of the low number of samples in this cohort.

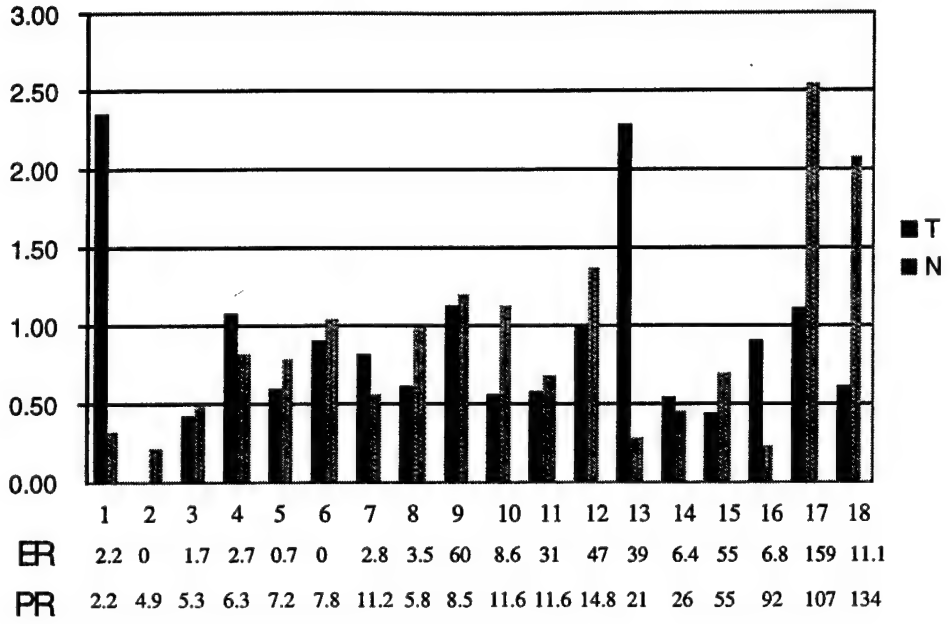

Figure 4

For each patient (1-18) normalized exon 3-deleted signal/WT-ER signal ratio (D3) is indicated for both normal (gray) and primary invasive lesion (black). ER and PR levels of the tumor, as determined by ligand binding assay, are also indicated.

Similar to what was seen using exon 5-deleted primer set, no signal was obtained for the tumor compartment of patient 2. A trend toward an higher expression of exon 3-deleted variant in the normal compartment is observed (11 cases out of 17), but this difference did not reach statistical significance. An increased number of samples would be required to confirm or refute this observation statistically.

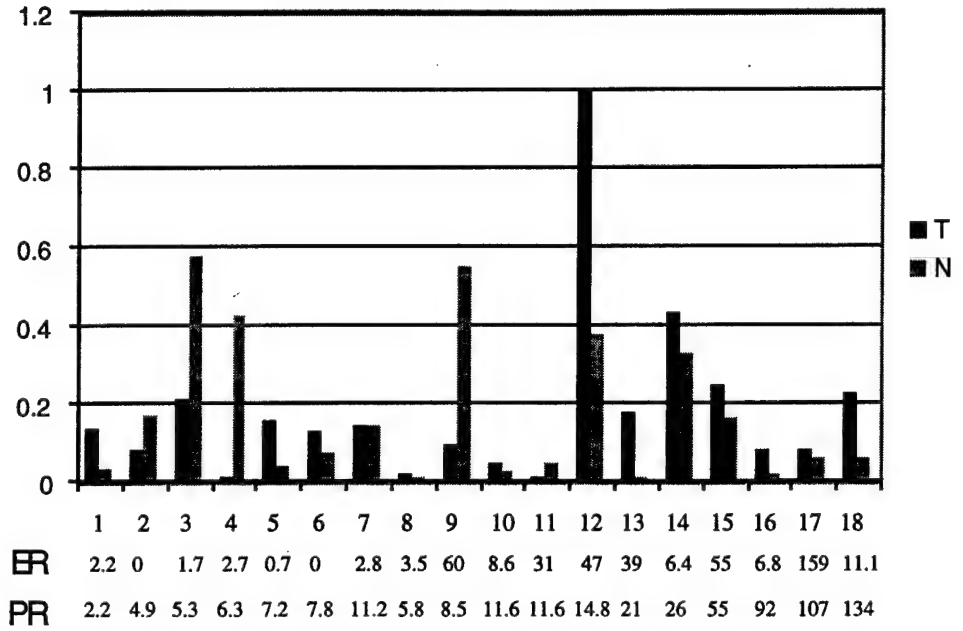

Figure 5

For each patient (1-18) normalized clone 4 signal/WT-ER signal ratio (C4) is indicated for both normal (gray) and primary invasive lesion (black). ER and PR levels of the tumor, as determined by ligand binding assay, are also indicated.

Considering the full cohort clone 4 mRNA expression was higher in the tumor component compared to the normal in 10 out of 18 cases. A statistically significant higher expression in tumor is observed when considering the ER+/PR+ cohort (n=9, $p<0.02$), only. This result is consistent with what has been previously demonstrated using independent unmatched breast tumor and normal breast tissue samples.[10] In this previous study, we indeed compared the relative expression of clone 4 mRNA variant between reduction mammoplasties and a ER+/PR+ breast tumor subset.

A manuscript reporting these results is in preparation

We addressed the question of the expression of ER–α variant mRNAs during breast cancer progression. Using the same RT-PCR assays, we examined the relative expression of clone 4, exon 5-deleted and exon 7-deleted ER–α variant mRNAs in 15 primary breast tumors and in their matched concurrent axillary lymph

node metastases. Overall, there were no significant differences between the primary tumors and the matched metastases. These data are in press in the British Journal of Cancer (Appendix 3).[15]

Objective 2

Using targeted PCR, all ER variants previously identified in breast tumors were detected in normal breast tissue (ie exon 2-, exon 3-, exon 4-, exon 5-, exon 7-deleted and clone 4 truncated). This suggested that multiple ER variant mRNAs are expressed in both normal and tumor breast tissues. We have recently developed a strategy to allow the investigation of known and unknown exon-deleted or inserted ER variant mRNAs in any one tissue sample as well as to determine possible changes in the relative expression of such variants amongst themselves and with respect to WT-ER transcript.[16] Briefly, cDNAs corresponding to all exon-deleted ER variants are amplified together with the WT-ER mRNA using primers annealing with exon 1 and exon 8 sequences. A competitive amplification occurs amongst all exon-deleted or inserted ER variant transcripts, that depends on their initial relative representation.

This approach has been used successfully to identify exon 3-4-deleted variant mRNA as differentially expressed between an estrogen sensitive cell line (T5) and an estrogen nonresponsive cell line (T5-PRF), obtained by chronically depleting T5 cells of estrogen in long term culture. The results of this study, have been submitted to the Journal of Clinical Endocrinology and Metabolisms (Appendix 4).

Long range PCR analyses still remains to be performed on the matched normal and tumor samples previously selected.

Objective 4

Long range PCR performed ER–β primers annealing with sequences in exon 1 and exon 8 of ER–β gene allowed us to identify an exon 5-6-ER–β variant in human breast tumors (appendix 5).[17] This observation was the first observation of ER–β variants in human tissues and we suggested that, as with ER–α variants, ER–β variants might also have a role in the mechanisms underlying hormonal progression in breast cancer. Since then, we have identified using targeted RT-PCR analysis of RNA extracted from normal as well as

tumor breast tissue two other ER–β mRNAs isoforms deleted in exon 5 or exon 6 (Appendix 6).[18] The existence of the putative proteins encoded by these variants remains to be determined.

The existence of similar as well as several other variants deleted in exon 8 sequences and putatively able to encode C-terminally truncated proteins has been documented by other groups (for a review, see Appendix 2). It is now clear that the ER–β signaling pathway, as with that of the ER–α, is complex and will likely include the interaction between several ER–β isoforms. Therefore, the balance between the ER–α and ER–β related molecules may be an important parameter to consider when studying the action of estrogens on both normal and neoplastic mammary tissue. It should be noted that due to the presence of the several ER–β variants identified so far and the uncertainty of the primary sequence of ER–β (Appendix 7)[19], the choice of the probes to detect any ER–α and or beta molecule is important.

Objective 5

In order to establish whether changes occur in the balance of ER–α and ER–β receptor during breast tumorigenesis, we designed a multiplex RT-PCR assay that allows the relative ER–α/ER–β content to be measured in small tissue samples, their. The region co–αmplified in the PCR reaction consists of a region encompassing exon 1 and exon 2 sequences of ER–β, and exon 2 and exon 3 for ER–α. This assay has been validated using spiked cDNA preparations and has been used to study the relative ER–α/ER–β expression in the subset of 18 normal breast tissues and their matched 18 breast tumor samples mentioned above. The data obtained have been published in Cancer Research (Appendix 8).[20] No differences in the ratio ER–α/ER–β were observed in the ER- cohort. However, a significantly ($p<0.02$) higher ER–α/ER–β ratio was observed in the ER+ tumors compared with that of their matched adjacent normal component. This increase was attributed to a significantly ($p<0.05$) increased ER–α mRNA expression, often in conjunction with a lower ER–β mRNA expression in the tumor compared with that of the normal component. Our results suggest that the role of ER–α and ER–β driven pathway and/or their interaction change during human breast tumorigenesis.

CONCLUSION

A higher expression of exon 5-deleted and clone 4 ER variant in the breast tumor component compared to the normal counterpart of matched samples was observed. This is consistent with previous observations made on independent samples. Although there was a trend towards a higher expression of exon 3-deleted ER–α variant in the normal component compared to the tumor component of the same matched cases, this difference did not reach statistical significance.

We have described the presence within normal breast as well as in breast tumor tissues of several variant forms of ER–β mRNA deleted in exon 5, exon 6 and in exon 5+6 sequences. The biological significance of the presence of these variants and of wild-type ER–β in breast tissue, in particular their role in estrogen/antiestrogen action, remains to be determined. We showed that a significantly ($p<0.02$) higher ER–α/ER–β ratio was observed in the breast tumors compared with their matched normal breats tissues and that this increase was attributed to a significant ($p<0.05$) increase in ER–α mRNA expression and a lower ER–β mRNA expression in the tumor compared with that of the normal component in some ER+ cases. Our results suggest that the role of ER–α and ER–β driven pathway and/or their interaction change during breast tumorigenesis.

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APPENDIX 1

1997, Ann. Med., 29, 221.

Oestrogen Receptor Variants and Mutations in Human Breast Cancer

Leigh C. Murphy, Etienne Leygue, Helmut Dotzlaw, Deborah Douglas, Amanda Coutts and Peter H. Watson[1]

Several oestrogen receptor variant and mutated mRNA species have been identified in human breast samples and cell lines. Over-expression and altered expression of some of these mRNAs have been correlated with breast tumourigenesis and progression. The following review focuses on the current knowledge available in the scientific literature with respect to the type and characteristics of oestrogen receptor variants and mutations that have been identified as occurring naturally in human breast tissues and cell lines.

Key words: breast cancer; mutations; oestrogen receptor.

(Annals of Medicine 29: 221–234, 1997)

Introduction

Oestrogens are major regulators of mammary gland development. However, oestrogens are also involved in the growth and progression of mammary cancers (1). The principal mechanism by which the effects of oestrogen are mediated in either normal or neoplastic target cells is via an initial interaction with the oestrogen receptor (ER). This protein is an intracellular ligand-activated transcription factor regulating the expression of several gene products, which ultimately result in target-tissue-specific oestrogen responses. The ER can be divided into several domains, labelled A–F, starting from the N-terminus (2, 3). Mutational analyses have defined several functional regions within each domain. The A/B region contains a cell- and promoter-specific, ligand-independent nonacidic transactivating function (AF-1), which may have a role in the agonist activity of the tamoxifen-like antioestrogens (4–6). The C domain contains two zinc finger motifs, which are responsible for the specific DNA-binding activity of the protein (2, 3). The C domain also contains an apparently constitutive dimerization domain (2). The D domain is thought to be a flexible hinge region but also contains a number of basic amino acids conserved in all receptors, which may have a role in nuclear localization and DNA binding (2). The E domain contains the ligand-binding domain, a ligand-dependent dimerization activity and a ligand-dependent nonacidic transactivating function (AF-2). The carboxy terminal F domain was originally thought to have no functional significance; however, more recent analyses suggest that it has a specific modulatory function on transcriptional responses to oestrogens and antioestrogens that is influenced by cell context (7). Upon oestrogen binding the receptor undergoes conformational changes resulting in its 'activation', so that it forms stable homodimers that bind tightly to specific nucleotide sequences called oestrogen-responsive elements, or EREs (2, 3). EREs are usually found in the promoter region of those genes the transcription of which is regulated by oestrogen. In this way oestrogen can alter the transcription of several genes that ultimately lead to DNA synthesis and proliferation of breast cancer cells.

However, the involvement of oestrogen in mammary tumour growth and progression is thought to involve, at some stages, perturbations of the ER signal transduction pathway, which are likely to contribute to tumour progression and the eventual development of hormone independence and a more aggressive phenotype (8–10). One mechanism underlying such perturbations could be alterations in the structure and therefore function of the ER itself. This review will focus on structural changes in the ER that have been identified as occurring naturally in human breast tissues and cell lines.

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Identification and Structure of ER Variant and Mutant mRNA Species

Molecular evidence for the potential existence of variant and/or mutant ER proteins has been obtained by analysis of ER-like mRNA in normal and neoplastic breast tissues. Many different types of ER-like mRNAs distinct from the wild-type ER mRNA have now been identified in several tissues and cell lines, including both normal and neoplastic human breast cells and tissues. It should be remembered, however, that few of these ER-like transcripts have been cloned and characterized from cDNAs representing full-length transcripts. Indeed little, if any, attention has been paid to the presence or absence of a 3′-untranslated region, a polyadenylation signal and a poly A tail. Given these caveats to interpretation, several different patterns of ER-like mRNA have been found or predicted, as described in the following.

Transcripts Containing Precise Single or Multiple Exon Deletions

Multiple ER-like transcripts have been identified that contain precise exon deletions (11–20). Several of the exon-deleted transcripts that have been described in the literature are shown in Figure 1. The majority of these have been identified by reverse transcription–polymerase chain reaction (RT–PCR) approaches, which by virtue of specific primer design have focused on small regions of the known wild-type ER mRNA. More recently, however, ER-like transcripts containing two or three entire exon deletions have been detected in cell lines and tissue samples (16–20) and amongst the deletion-type ER variants, this type of variant ER transcript appears now to be the most predominant. However, the identification of multiple types of exon deleted transcripts in any one cell line or tissue sample (16–18, 20) underscores the need to study these variant ER transcripts altogether, as well as individually.

Other Deleted Transcripts

ER-like transcripts containing variable-sized deletions that are not entire exon deletions have also been detected. This type of alteration falls into two groups: one in which a single nucleotide has been deleted (21, 22), and the other in which several hundreds of continuous nucleotides have been deleted but starting and ending within known exon sequences (Fig. 1, Table 1) (16, 20, 21, 23).

Truncated Transcripts

These altered ER-like transcripts are significantly smaller than the wild-type ER mRNA as determined by Northern blot analysis (24). cDNA cloning of apparently full-length or near to full-length transcripts was used to characterize these transcripts fully. These transcripts contain entire exon sequences of at least 2 of the 5′ ER exon sequences, and then diverge into ER-unrelated sequences (25), some of which appear to be LINE-1

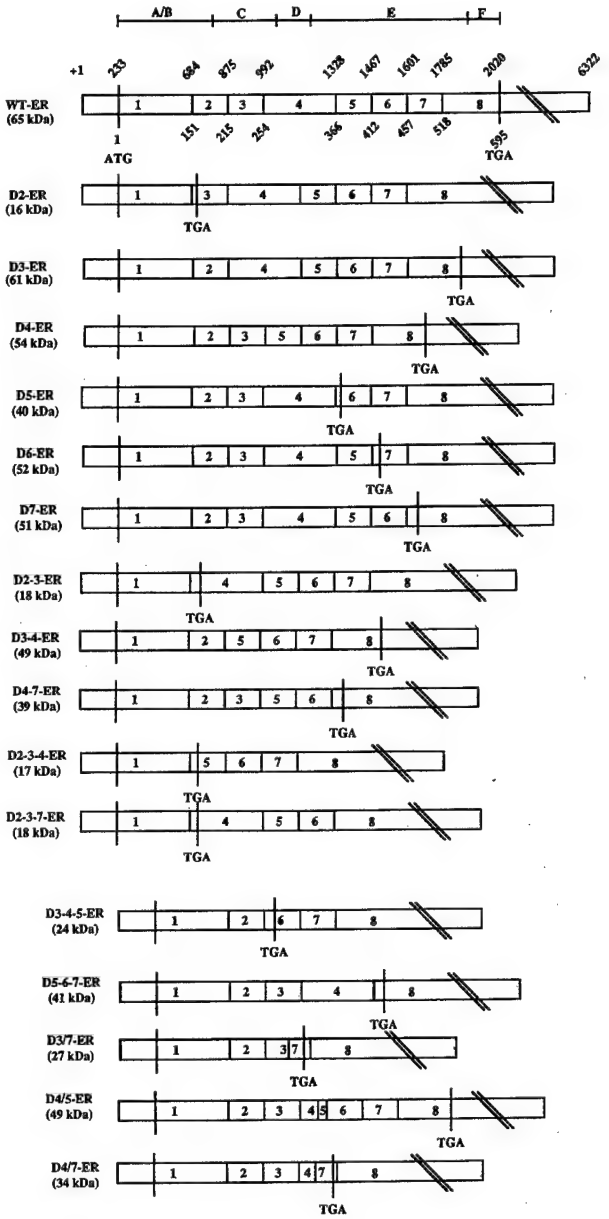

Figure 1. Schematic diagram of the wild-type human oestrogen receptor (WT-ER) cDNA, which contains eight different exons coding for a protein divided into structural and functional domains (A–F). Region A/B of the receptor is implicated in transactivating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another transactivating function (AF-2). The numbering on the top of the cDNA refers to the nucleotide position as defined in (64). Below the WT-ER cDNA are the various putative exon and other large deleted ER cDNAs. ATG shows the translation initiation codons, TGA shows the inframe translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in (64). D indicates deletion, and the estimated molecular mass (kDa = kiloDaltons) of each open reading frame is shown in parentheses. Molecular masses were estimated using MacVector version 4.1.4 software.

Table 1. ER variants identified in human breast tissues and cell lines.

Variant mRNA	Estimated M_r of predicted protein (kDa)	Functional domains	Reference
Wild-type ER	65	A, B, C, D, E, F	(4, 5, 64)
D2-ER	16	A, B?	(11, 16–19)
D3-ER	61	A, B, D, E, F	(11, 14)
D4-ER	54	A, B, E?, F	(12, 17–20, 59)
D5-ER	40	A, B, C, D	(13)
D6-ER	52	A, B, C, D	(14)
D7-ER	51	A, B, C, D	(11, 15, 20)
D2-3-ER	18	A, B?	(16, 20)
D3-4-ER	49	A, B, E?, F	(16, 17, 20)
D4-7-ER	39	A, B	(18–20)
D2-3-4-ER	17	A, B?	(20)
D2-3-7-ER	18	A, B?	(20)
D3-4-5-ER	24	A, B	(18)
D5-6-7-ER	41	A, B, C	(18)
D3/7-ER	27	A, B	(20)
D4/5-ER	49	A, B, C?, F	(21)
D4/7-ER	34	A, B, C?	(23)
Clone 4-ER	24	A, B	(25)
Clone 24-ER	37	A, B	(25)
Exon 6^2-ER	51	A, B, C, D	(26)
Exon $(34)^2$-ER	75	A, B, C+, D+, E, F	(26)
Exon $(67)^2$-ER	80	A, B, C, D, E+, F	(27, 45)
ER-69-bp	69	A, B, C, D, E?, F	(26, 28)

? Indicates that an alteration of the function has been shown or is likely to occur.

related (Fig. 2, Table 1). Although several different truncated ER mRNAs have been cloned, some of these were only found to be expressed in a single breast tumour, although others, such as the clone 4-truncated ER mRNA, have been found to be expressed in many human breast tumours (25).

Insertions

ER-like transcripts have been identified containing variable-sized nucleotide insertions. Such insertions consist of one to two nucleotides (21, 22), larger insertions of 69 and more nucleotides (21, 26), and apparently complete exon duplications (26, 27) (Fig. 2, Table 1). These abnormal ER-like transcripts were detected using RT–PCR analyses, and further studies showed that the exon 6 plus 7-duplicated ER-like transcript was generated from a mutated ER gene in which genomic rearrangement resulted in the duplication of exons 6 and 7 in an in-frame fashion (27). As well the 69-bp-inserted ER mRNA is probably generated from a point mutation in one allele of the ER gene in the breast tumour from which it was cloned. This point mutation generates a consensus splice donor site at the 3′ end of the 69-bp sequence present in intron 5. In addition, a splice acceptor consensus sequence is normally present at the 5′ end of the 69-bp sequence, and thus the 69 sequences are likely to be seen as another exon in the gene (28).

Point Mutations

Several point mutations including silent polymorphisms have been identified in ER-like transcripts (Table 2) (21,

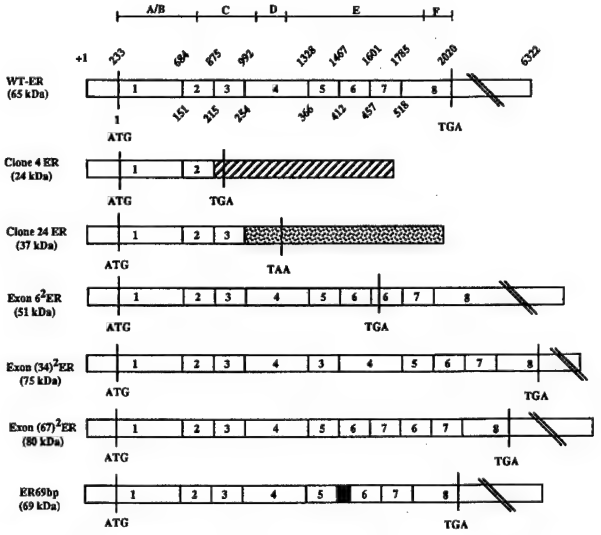

Figure 2. Schematic diagram of the wild-type human oestrogen receptor (WT-ER) cDNA, which contains eight different exons coding for a protein divided into structural and functional domains (A–F), as described in Figure 1. The numbering on the top of the cDNA refers to the nucleotide position as defined in (64). Below the WT-ER cDNA are the clone 4- and clone 24-truncated ER cDNAs, which have been cloned previously (25), as well as the putative cDNAs representing exon-duplicated and some inserted ER mRNAs (26, 27). ATG shows the translation initiation codons, TGA shows the in-frame translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in (64). The estimated molecular mass (kDa = kiloDaltons) of each open reading frame is shown in brackets. Molecular masses were estimated using MacVector version 4.1.4 software.

Table 2. Small insertions/deletions and point mutations/polymorphisms identified in the oestrogen receptor mRNA.

Nucleotide change	Exon	Amino acid change	Functional domains	Reference
262 T→C	1	10 Ser no change	A, B, C, D, E, F	(25, 29)
439 C→G	1	69 Asn→Lys	A, B?, C, D, E, F	(29)
493 G→C	1	87 Ala no change (B variant)	A, B, C, D, E, F	(29–32)
701 C→T	2	+Stop after 156	A, B?	(36)
961 C→T	3	243 Arg no change	A, B, C, D, E, F	(29)
TT insert after 981	3	Met 250→Ile+stop	A, B	(21)
1059 C→T	4	276 Gly no change	A, B, C, D, E, F	(22)
1119 T→C	4	296 Leu→Pro	A, B, C, D?, E?, F	(32)
1207 C→G	4	325 Pro no change	A, B, C, D, E, F	(29, 32)
1283 G→T	4	352 Asp→Tyr	A, B, C, D, E?, F	(34, 35)
1290 A→T	4	353 Glu→Val	A, B, C, D?, E?, F	(22)
1418 A→G	5	396 Met→Val	A, B, C, D, E?, F	(29)
1463 G del	5	411 Asp→Thr+6 extra novel a.a.	A, B, C, D	(21)
del T at 1526	5	432 Ser→His+4 extra novel a.a.	A, B, C, D	(22)
1503–1550 replaced	6	424 Ile→Arg+28	A, B, C, D	(22)
by 1380–1422	5	extra novel a.a.		
1647 G→A	7	472 Lys no change	A, B, C, D, E, F	(22)
1747 C→G	7	505 Ala no change	A, B, C, D, E, F	(22)
1963 T→C	8	577 His no change	A, B, C, D, E, F	(22)
2014 A→G	8	594 Thr no change	A, B, C, D, E, F	(29)

Nucleotides are numbered according to the start site of transcription (+1) in (64). ? Indicates that an alteration of the function has been shown or is likely to occur.

22, 29–35). The only known germline mutation in the human ER associated with disease is a point mutation (36) identified in a young adult male presenting with osteoporosis, unfused epiphyses, continued linear growth in adulthood, and oestrogen resistance. Furthermore, only approximately 1% of primary breast tumours have point mutations in the ER gene (22, 29), which in some cases might be linked to hereditary breast cancer (37).

The above ER-like mRNA molecules have, in most cases, been identified in human breast cancer tissues or human breast cancer cell lines. However, data are now emerging showing that several of the exon-deleted and truncated transcripts are also expressed in multiple samples of normal human breast tissue (16–18). This suggests that the mechanisms for generating these transcripts are present in normal human mammary cells and therefore these transcripts are normal variants, and probably generated by an alternative splicing mechanism (38). It is less likely that the inserted transcripts and many of the amino acid altering point mutations are normal variants. There is a greater likelihood that such transcripts were generated from a mutated ER allele present in some human breast tumours (27, 28). In summary, a large body of molecular data exists to support at least the potential for the existence of variant or abnormal ER-like proteins in human breast cancer.

Expression of Multiple ER Variant mRNAs in Human Breast Tissues

The identification of several ER variant mRNAs in normal human breast tissues implies that either the variant mRNAs or their respective proteins may have a normal role in ER signal transduction. Consequently, changes in the balance of ER-like molecules could perturb the ER signalling pathway and contribute to tumour progression. It has therefore become important to determine whether levels and the pattern of ER variant expression are different between normal and neoplastic breast tissues, as well as amongst groups of tumours with different characteristics.

This has been studied initially by investigating individual variant ER mRNA levels relative to wild-type ER mRNA levels. The relative expression of the truncated clone 4 ER mRNA (39) and the exon 5-deleted ER mRNA, but not the exon 7-deleted ER mRNA (16) were found to be significantly elevated in breast tumour tissue compared with normal breast tissue. It has also been suggested that the level of the exon 3-deleted ER mRNA is reduced in breast tumour tissue compared with normal tissue (40). Such data suggest that the expression of some but not all variant ER mRNAs is deregulated during breast tumourigenesis.

Investigation of the relative expression of the truncated clone 4 ER variant in groups of breast tumours with different prognostic characteristics (41) identified a statistically significant increased expression of this transcript in breast tumours with combined characteristics of poor prognosis (node positive, large tumour size, high S-phase fraction) and lack of endocrine sensitivity (progesterone receptor (PR) negative). Elevated exon 5-deleted ER transcripts have been found in ER−/PR+ and ER−/pS2+ tumours (42), while increased levels of the exon 7-deleted ER mRNA are often found in ER+/PR− breast tumours (15).

These data suggest that altered expression of some ER variants is associated with different phenotypes in

human breast tumours and may have a functional role in such phenotypes. However, it has become increasingly apparent that several ER variant mRNAs can be detected in any one sample of either normal or cancerous breast tissues (16–18). While it is unclear whether any or all of these mRNAs are stably translated *in vivo* (see discussion below), many of the predicted ER-like proteins are lacking some functional domains (4) of the wild-type ER (Figs 1 and 2), and some have been shown to exhibit altered functions *ex vivo*. Therefore, the possibility exists that several ER variant proteins could be expressed together (16–18) and the validity of investigating individual variants in isolation can be questioned. Furthermore, previous analyses have depended largely on assays that focus on limited regions of the transcript, and would be unlikely to detect more than one modification per individual variant mRNA. However, it is now clear that more than one modification can occur in variant transcripts (19). Thus signals attributed to the exon 7-deleted ER variant mRNA, detected by RT–PCR using primers in exon 5 and 8 or by RNAse protection assays with probes covering the exon 6/8 junction, may also include contributions from a variant deleted in both exon 4 and 7, recently identified by Madsen et al. (19). Nevertheless, these molecules may result in quite different proteins that differ in activity and may modulate differentially the ER signalling pathway. There is thus a need to investigate qualitatively and quantitatively the expression of total ER variant mRNAs within a single tumour. An attempt to address this issue was published recently (20). A strategy was developed to allow the investigation of known and unknown exon-deleted or inserted ER variant mRNAs in any one tissue sample as well as to determine possible changes in the relative expression of such variants amongst themselves and with respect to the wild-type ER transcript. The approach (20) used is illustrated in Figure 3; however, owing to practical limitations it cannot measure all types of ER variants, and indeed the truncated transcripts would not be included in such an analysis (25, 39). A competitive amplification occurs amongst all exon-deleted or inserted ER variant transcripts, which depends on their initial relative expression, and the detection of bands corresponding to specific ER variants reflects the relative expression of these ER variant mRNA species within the samples. A survey of 100 breast tumours (20), showed that the most frequently expressed ER variants at a relatively high abundance were the exon 7-deleted variant, the exon 4-deleted variant, a variant deleted in both exons 3 and 4, a variant deleted in exons 2, 3 and 7, a variant deleted in both exons 4 and 7, a variant deleted in exons 2, 3 and 4, and a variant deleted from within exon 3 to within exon 7. Neither the exon 5-deleted nor the exon 3-deleted ER mRNAs were detected using this approach. Interestingly, preferential detection of some deleted variants was found to be associated with known prognostic markers in breast cancer (20).

In summary, data exist to support the hypothesis that altered expression of variant ER mRNA expression occurs during both breast tumourigenesis and breast cancer progression.

Expression of Variant or Mutant ER Proteins

It is unclear at this stage whether all or any of the ER-like transcripts so far identified are stably translated *in vivo*. It is certainly possible for many of them to be expressed at high levels from expression constructs transfected into mammalian, yeast and bacterial host cells. Furthermore, in some cases ER variant expression under these conditions has identified a putative function of the resulting variant protein (11, 13, 15). For example, exon 3 and exon 7-deleted variants may act as dominant negative regulators (inhibitors) of wild-type ER (11, 40) whereas exon 5-deleted ER has ligand-independent transcriptional activity (13, 43) (see discussion below).

More importantly, an ER-like protein consistent with that predicted to be encoded by the exon 5-deleted ER transcript has been found to be expressed naturally in some BT-20 human breast cancer cell lines (44). In addition, an immunoreactive 80 kDa ER-like protein has

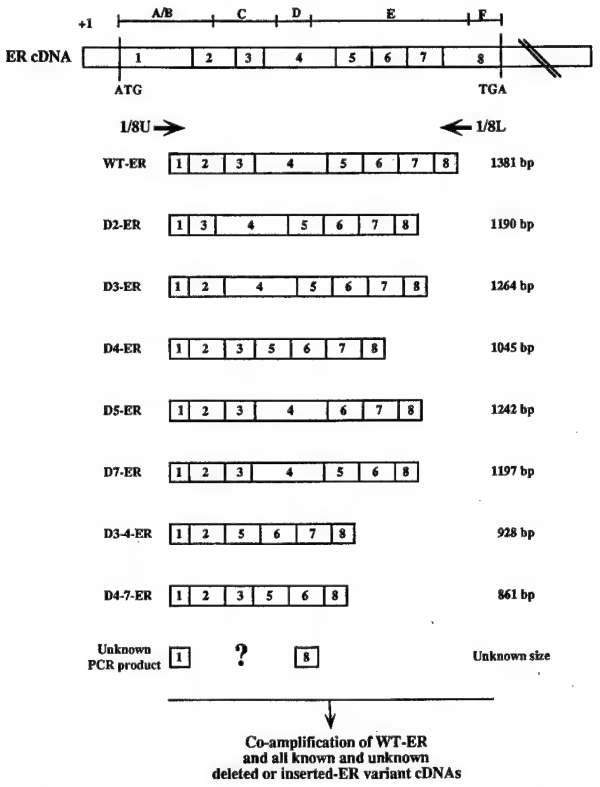

Figure 3. Schematic representation of wild-type oestrogen receptor (WT-ER) cDNA and primers allowing coamplification of most exon-deleted ER variants. 1/8U and 1/8L primers allow amplification of a 1381 bp fragment corresponding to WT-ER mRNA. Coamplification of all possible exon-deleted or inserted variants that contain exon 1 and 8 sequences can occur (20). Amplification of the previously described ER variant mRNAs deleted in exon 2 (D2-ER), exon 3 (D3-ER), exon 4 (D4-ER), exon 5 (D5-ER), exon 7 (D7-ER), both exons 3 and 4 (D3-4-ER), exons 2 and 3 (D2-3-ER) and exons 4 and exon 7 (D4/7-ER) would generate 1190 bp, 1264 bp, 1045 bp, 1242 bp, 1197 bp, 928 bp, 1073 bp and 861 bp fragments, respectively.

been identified in an MCF-7 subclone (45). This protein corresponds to the predicted protein encoded by an ER-like transcript containing an exon 6 and 7 duplication, which was cloned from the same cell line (27). These data demonstrate the ability of some ER-like transcripts to be naturally translated into stable proteins, which can be detected by current methods, and suggest the likelihood of other ER-like transcripts being stably translated *in vivo* under natural conditions.

Other studies support the expression of variant or mutant ER-like proteins but their relationship to known variant or mutant ER mRNA remains unclear. Immunohistochemical staining with a polyclonal antibody was used previously to identify two types of apparently defective ER in human breast cancers (46); one that bound the nucleus in a ligand-independent fashion and one that could not bind to the nucleus even in the presence of ligand. Several other studies have identified ligand-binding forms of the ER that have both altered molecular mass (often truncated compared to the wild-type ER) and altered isoelectric points (47). The correlation of some of these ER-like proteins with biological parameters suggests that they may play a role in the ER signal transduction pathway (48). More recently, truncated DNA-binding forms of ER-like proteins have been identified in some human breast cancer biopsy samples (49). ER antibodies (Fig. 4) recognizing epitopes in the A/B and E domains of the wild-type receptor were found to detect these truncated ER-like proteins. An ER-like protein was identified in some ER+/PR− human breast tumours that formed complexes with an oligonucleotide containing an ERE in gel shift assays (15). The complex was supershifted by H226 and H222 antibodies but not by the D75 antibody recognizing a more C-terminal epitope (Fig. 4). Steroid hormone-induced mammary tumours in Grunder mice progress from hormone dependence to hormone independence following serial transplantation. This progression is associated with decreased expression of the 65 kDa ER protein and a marked increase in tamoxifen aziridine-bound, immunopurified 50 and 35 kDa proteins (50).

The relationship of any of these ER-like proteins that have been characterized in some human and mouse tumour tissues to ER-like proteins that are potentially encoded by some of the previously characterized ER-like mRNAs is unclear and remains to be elucidated. Very few, if any, Western blotting analyses using differential antibody detection of ER-like proteins in human breast tumours have been reported. One study, where ER antibodies recognizing epitopes within the ligand-binding domain were used for Western blotting, identified larger than wild-type as well as smaller than wild-type ER immunoreactive proteins (49, 51). However, many of the known variant ER transcripts are likely to encode proteins around the size of heavy and light immunoglobulin chains. Immunoglobulin contamination of human breast tumours and immunoprecipitated complexes would probably interfere with Western blot analysis of such variant ER proteins (51).

More recently, a group of human breast tumours were analyzed immunohistochemically (52) for ER expression by using antibodies that recognize either an N-terminally localized epitope in the wild-type ER protein, or a C-terminally localized epitope in the wild-type ER protein (Fig. 4). It was found that the antibody recognizing the C-terminally localized epitope correlated better with the ligand-binding assays performed on adjacent tissues than did the antibody recognizing the N-terminally localized epitope. Additionally, although in many tumours the immunohistochemical results using each antibody showed good concordance, in some tumours the results were discordant, with the signal tending to be higher with the N-terminal antibody (53). Because many of the proteins predicted from variant ER mRNAs would be truncated at the C-terminus and would not contain the epitope recognized by the C-terminal antibody, one interpretation of these data would be that truncated variant ER proteins are more highly expressed in the discordant group of tumours. This hypothesis was tested by investigating the pattern and relative expression of variant ER mRNAs in the discordant and concordant groups of breast tumours. Several ER variant mRNAs that encode putative short ER-like proteins that would be recognized only by an N-terminal-targeted antibody were preferentially and more highly expressed in the discordant breast tumour group. These ER variants were: the clone 4-truncated ER mRNA; the exon 2, 3 plus 7-deleted ER mRNA; the exon 2, 3 plus 4-deleted ER mRNA; and the variant deleted within exon 3 to within exon 7 (53). The data suggest that the ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER measured by immunodetection assays using N- or C-terminal antibodies. Further, the data are consistent with the ability of ER variant mRNAs to be stably translated *in vivo* and therefore have a functional role or roles in ER signal transduction.

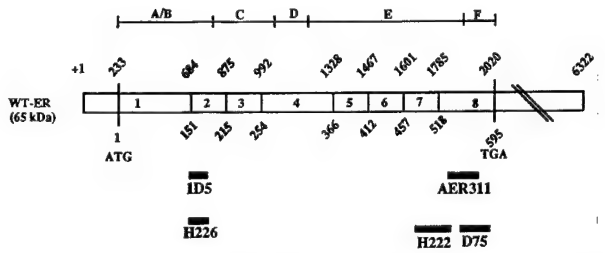

Figure 4. Approximate location of the epitopes recognized by the various oestrogen receptor antibodies (1D5, H226, H222, AER311, D75) referred to in this review. Schematic diagram of the wild-type human oestrogen receptor (WT-ER) cDNA, which contains eight different exons coding for a protein divided into structural and functional domains (A–F). The numbering on the top of the cDNA refers to the nucleotide position as defined in (64) and the numbering below the cDNA refers to the amino acid positions as defined in (64).

Structure and Function *In Vitro* and *In Vivo* of Putative Variant and Mutant ER-like Proteins

The data summarized in the previous section suggest the likelihood of some or all ER-like transcripts, being

stably translated *in vivo*. This provides a rationale for discussing the possible structure and function of the proteins predicted to be expressed from variant and/or mutant ER-like mRNAs.

1. Exon 7-deleted ER

Relative to all other deleted ER transcripts the exon 7-deleted ER variant appears to be the most abundant in human breast tissues (20). This transcript was first identified in T-47D human breast cancer cells (11) and was subsequently found in human breast tumour samples (15). The predicted protein encoded by this variant ER transcript is approximately 51 kDa (Fig. 1, Table 1), and is identical to the wild-type ER protein up to amino acid residue 456 and thereafter encodes 10 novel amino acids. The putative protein encoded by the exon 7-deleted transcript would therefore be truncated in the E domain, which includes the ligand binding, AF-2 and a strong dimerization domain of the wild-type receptor. The putative function of the protein encoded by the exon 7-deleted transcript is controversial. Wang and Miksicek (11) using HeLa cells found that it did not bind ERE DNA or have transcriptional activity of its own. Moreover, under these conditions the exon 7-deleted ER did not affect the activity of the wild-type ER. These data contrast with those obtained by Fuqua et al. (15), where, using a yeast expression system, the exon 7-deleted ER protein was found to inhibit wild-type ER activity. Furthermore, Fuqua et al. had originally isolated the exon 7-deleted ER variant mRNA from an ER+/PgR− breast tumour that contained an ER-like protein able to bind to DNA containing an ERE as determined by gel mobility shift analysis, but that interacted differentially with ER antibodies suggestive of an ER-like protein with a C-terminal truncation (15). The data of Fuqua et al. were consistent with the idea that overexpression of an exon 7-deleted ER protein could contribute functionally to the ER+/PR− breast tumour phenotype. The hypothesis was further supported by the observation that exon 7-deleted mRNA levels were significantly elevated in a group of human breast tumours that were ER+/PgR−/pS2− compared to those which were ER+/PgR+. Although exon 7-deleted mRNA was found in normal breast tissue, its expression was not significantly different in normal versus breast tumour tissue, although in the same tissue samples the levels of both the exon 5-deleted and the clone 4-truncated ER mRNAs were significantly higher in tumours compared to normal breast tissues (16). This latter study, in contrast to studies reported by Fuqua et al., did not find any significant relationship between exon 7-deleted ER mRNA level and PR status or tumour grade (16, 20).

The reported data suggest that the activity of the exon 7-deleted ER may vary in a cell-type and promoter-specific fashion. This in turn suggests that the background milieu may dictate the impact of variant ERs. So, although there are a few reports of altered exon 7-deleted ER expression, it is one of the most abundantly expressed variants in human breast tissues and activity of this variant may depend on an altered cellular milieu.

2. Exon 5-deleted ER

The predicted protein from the exon 5-deleted ER mRNA is a truncated protein of approximately 50 kDa as a stop codon has been introduced after amino acid residue 371 (Fig. 1, Table 1). Its amino acid composition would be identical to the wild-type ER up to amino acid 366 followed by five novel amino acids, and consequently the majority of the ligand-binding domain of the wild-type ER will be missing.

Using a yeast expression system Fuqua et al. (13) showed that the exon 5-deleted ER displayed ligand-independent transcription from an ER-regulated reporter plasmid. Rea and Parker (54) confirmed this result in chicken embryo fibroblasts. However, when an exon 5-deleted ER expression vector was stably transfected into MCF-7 cells, it had no effect on an ERE-tk-luciferase reporter plasmid, it slightly increased transcription from an ERE_2TATA-CAT but not an ERE_1TATA-CAT reporter plasmid, and it had no effect on endogenous oestrogen-responsive genes such as pS2 and progesterone receptor. Neither did it result in the development of oestrogen independence and antioestrogen resistance in these cells. In contrast, a similar study by Fuqua and Wolf (55) showed that over-expression of the exon 5-deleted ER protein resulted in increased progesterone receptor levels in the absence of oestrogen, as well as oestrogen-independent growth and tamoxifen resistance. The reasons for the different results between the two groups are unclear, although differences in the original parent MCF-7 cells was suggested, in turn suggesting that other changes in addition to altered exon 5-deleted ER expression are required for hormonal progression in human breast cancer cells. This is not unreasonable as several mechanisms, either alone or in combination, may be responsible for such progression (10). For example, it is possible that the alteration of growth factors or their cognate receptors, some of which have been shown to result in ligand-independent activation of the wild-type ER through the N-terminal AF-1 domain (56, 57), may also be required in conjunction with altered expression of ER variants. Interestingly, Klotz et al. (58) identified a correlation between increased expression of the exon 5-deleted ER transcript relative to the wild-type ER and reduced responsiveness to oestrogen in MCF-7 stocks obtained from various laboratories in North America.

Measurement of the exon 5-deleted ER mRNA in clinical samples provides further insight into a possible role for this ER variant. The exon 5-deleted ER transcript was found to be present in normal human mammary tissue, but its level relative to the wild-type ER mRNA was significantly increased in breast tumour tissues (16). The exon 5-deleted ER transcript was first identified in a tumour that was ER−/PgR+, a finding consistent with the speculation concerning the ligand-independent activity of a protein encoded by this transcript (13). Using a specific RT–PCR approach, this transcript has been found to be more highly expressed,

and in some cases more abundant, than the wild-type ER mRNA, in ER−/PgR+ breast tumours (13, 42). However, when measured within a wide range of ER+/PR+ breast tumours, using a long-range RT–PCR approach (20), its relative expression with respect to all other deleted transcripts is low to undetectable (20, 59). Again using a specific RT–PCR analysis, Daffada et al. (42) found significantly higher levels of the exon 5-deleted ER transcript in those human breast tumours that were ER−/PgR+ or ER−/pS2+. However, while levels of the exon 5-deleted ER transcript are found to vary widely in human breast tumours, no significant differences in their levels were found between tamoxifen-resistant and tamoxifen-sensitive tumours (42). Furthermore, in a tamoxifen-resistant MCF-7 cell line the level of the exon 5-deleted transcripts was lower than the sensitive parent line (19) although differential expression of other ER variants was found. Presently no clear-cut correlation between exon 5-deleted ER expression and tamoxifen resistance is evident. However, this might be expected because there are multiple variants expressed in any one tumour and multiple mechanisms are likely to be involved in the development of tamoxifen resistance in particular and endocrine therapy resistance in general (10).

In conclusion, the putative activity of the protein encoded by the exon 5-deleted ER mRNA could contribute to the development of oestrogen independence and endocrine resistance in human breast cancer. Certainly correlations between the level of this transcript and apparently constitutively elevated oestrogen target gene expression would support this hypothesis. However, differences between the phenotypes generated by stable transfection studies and the lack of correlation of this transcript with tamoxifen-resistant breast tumours suggest that other factors are probably involved, either together with or independently of elevated exon 5-deleted ER expression. Although the naturally occurring exon 5-deleted ER mRNA and its putative cognate protein have been the most widely studied ER variants to date, we now know that multiple ER variants can be found in both normal and neoplastic breast tissues (16–18). Furthermore, using assays that allow the investigation of the relative expression of multiple ER variant mRNAs, it is apparent that the exon 5-deleted transcript represents one of the lower abundance variant transcripts in a wide range of human breast tumours, except perhaps in the ER−/PR+ phenotype.

3. *Exon 4-deleted ER*

The exon 4-deleted ER transcript has been found expressed in human breast cancer cell lines (12, 19, 59), human breast cancer tissue (17, 18, 20) and normal human breast (17, 18). This transcript contains an in-frame deletion and is predicted to encode a protein of approximately 54 kDa (Fig. 1, Table 1) which would be missing a strong nuclear localization domain and a portion of the E domain of the wild-type ER. When an expression vector was made for this variant, the encoded protein did not bind oestradiol or an ERE, and had no transcriptional activity of its own nor any dominant negative activity against the wild-type ER (61, 62). Although these studies suggest that an exon 4-deleted ER is essentially inactive, negative results may reflect the promoter and cell types used in these studies. More recently, a correlation was found between the relatively increased expression of the exon 4-deleted ER mRNA with high PR expression and low grade, suggesting its correlation with some good prognostic features in human breast tumours (20). However, any functional role that this ER variant might have in this correlation is as yet unclear.

4. *Exon 3-deleted ER*

An exon 3-deleted ER transcript was initially identified in T-47D human breast cancer cells (11). A deletion of exon 3 from the wild-type ER transcript is in frame and generates a protein of approximately 61 kDa that lacks the second zinc finger of the wild-type ER DNA-binding motif. The function of the putative protein encoded by this transcript is controversial. When expression vectors for this protein were transfected into HeLa cells the exon 3-deleted protein demonstrated a dominant negative activity, inhibiting wild-type ER transcriptional activity, without any intrinsic transcriptional activity of its own. This variant ER, while unable to bind to an ERE in a gel mobility shift assay, inhibited the ability of the wild-type ER to bind to an ERE under the same conditions (11, 14). Preliminary data in which this variant was stably over-expressed in MCF-7 human breast cancer cells suggest that it has dominant negative activity in this model as well. Over-expressing cells were growth-inhibited by oestrogen, suggesting that this variant can inhibit the mitogenic effect of oestrogen in these cells (40). In contrast, in a yeast expression system this variant ER does not have transcriptional or dominant negative activity (63).

Again the data available in the literature concerning the potential activity of the exon 3-deleted ER suggest that variant activity as well as wild-type ER activity can depend on the gene promoter used and the cellular milieu. Interestingly, reported in abstract form is the observation that the level of the exon 3-deleted ER transcript is higher in normal mammary epithelia compared to breast tumours and tumour cell lines (40). This raises the interesting possibility that the expression of this variant may decrease with breast tumourigenesis and the exon 3-deleted ER may have an important role in the control of ER signalling and the control of breast epithelial cell growth. In a range of 100 breast tumours, using an approach that allowed the investigation of the relative expression of multiple ER-deleted mRNAs, the exon 3-deleted variant transcript was low to undetectable (20), while another study suggested that the level of this variant was similar in all ER+ breast tumours and was therefore unlikely to be involved in the evolution of the ER+/PgR− breast cancer phenotype in contrast to the exon 7-deleted variant (63). However, no compari-

son with normal human breast tissue was made in either of these two latter studies.

5. *Exon 2-deleted ER*

An ER-like transcript deleted in exon 2 sequences was first demonstrated in T-47D human breast cancer cells (11). Subsequently, it was identified in MCF-7 cells (19) and both normal (16–18) and neoplastic breast tissues (16–18). The exon 2-deleted transcript could encode a truncated protein of approximately 16 kDa missing the entire DNA- and ligand-binding domains (Fig. 1, Table 1). The protein would only encode the A/B region of the wild-type ER up to amino acid 151 with an additional novel amino acid residue. The protein encoded by this transcript displayed no transcriptional activity of its own, but exhibited a mild dominant negative activity when over-expressed at least 20-fold relative to the wild-type ER protein (11). This transcript was found to be over-expressed in a tamoxifen-resistant MCF-7 cell line compared to the parent MCF-7 cells, although other ER variant transcripts were also differentially expressed in these two cell lines (19). Although such data support a role for altered ER variant expression in hormone independence, the mechanism or mechanisms by which this is achieved is unknown.

6. *Multiple-exon-deleted and Other Deleted ERs*

Several multiple-exon-deleted ER transcripts have recently been identified in human breast cancer cells (19), and in both normal and neoplastic human breast tissues (16–18, 20). These include both double- and triple-exon deletions. Deletions of exons 4 and 7 from the one transcript have been described in human breast cancer cells (19) and human breast tissue (18). Furthermore, this transcript is frequently expressed at a relatively high level in a wide range of human breast tumours (20). An exon 4- and 7-deleted ER transcript is predicted to encode a protein of approximately 39 kDa (18) deleted in the hinge region, lacking a nuclear localization signal and significant portions of the ligand-binding and AF-2 domains. No studies reporting putative function have been published. Leygue et al. (16, 20) have identified transcripts deleted in exons 2 and 3, and transcripts deleted in exons 3 and 4 in human breast tissues. This latter transcript was also identified in human breast tissues by Gotteland et al. (17). The transcript is predicted to encode an inframe protein of approximately 49 kDa, lacking ER amino acid residues encoded on exons 3 and 4, i.e. amino acids 216–365 (Fig. 1, Table 1). This protein would be unable to bind to DNA, would be missing a nuclear localization signal and part of the hormone-binding domain. Interestingly, the relative expression of this transcript is increased markedly in human breast cancer cells that have become oestrogen independent (A. Coutts, E. Leygue and L. Murphy, unpublished observation). No data are available with regard to the potential function of this protein in human breast cells, although the protein encoded by this transcript has no transcriptional or dominant negative activity in a rat aortic smooth muscle cell line model (61).

Triple-exon-deleted ER transcripts have been observed in MCF-7 human breast cancer cells (18) and in human breast cancer tissue (20). MCF-7 cells contain ER transcripts deleted in exons 3, 4 and 5 as well as transcripts deleted in exons 5, 6 and 7 (18). Leygue et al. (20) recently identified in human breast cancer tissues ER transcripts deleted in exons 2, 3 and 4 and exons 2, 3 and 7. No functional studies have been carried out on the proteins encoded by such transcripts; however both these transcripts were frequently detected at a relatively high level of expression in a wide range of human breast tumours (20). In addition, the detection of the exon 2, 3 and 4-deleted transcript was significantly correlated with high-grade tumours (20).

Deletions that are not exact exon deletions have also been described. Grahame et al. (21) identified in T-47D cells an ER-like transcript deleted of 462 bases from within exon 4 to within exon 5. This predicts for a putative protein containing 442 amino acids with an in-frame deletion of 153 amino acids of the wild-type ER protein (Fig. 1, Table 1). The predicted protein is deleted from the end of the DNA-binding domain to mid-ligand-binding domain. This same group observed an ER transcript deleted in a G residue (nucleotide 1463) (numbered according to (64) of wild-type ER sequence) at amino acid residue 411 in the hormone-binding domain of the ER. This resulted in a frame shift so that a truncated protein is encoded (Table 2). The predicted protein is identical to the wild-type ER up to amino acid residue 410, followed by seven novel amino acids. The protein would have an intact DNA-binding domain and hinge region but would be truncated in the ligand-binding domain. Similarly, Karnik et al. (22) identified an ER-like transcript in a tamoxifen-resistant metastatic human breast tumour that was deleted in a T residue in exon 6. This would generate a frame shift resulting in a protein identical to the wild-type ER up to amino acid residue 433 followed by five novel amino acids (Table 2). This protein is probably defective in its ligand-binding and AF-2 activities. Daffada and Dowsett (23) described a novel splice variant of the ER mRNA in normal human endometrial tissue and breast cancers. This variant consists of a deletion within exon 4 sequences to within exon 7 sequences. This variant is out of frame, is identical to the ER up to amino acid residue 277 and thereafter encodes another 32 novel amino acids (Fig. 1, Table 1). The predicted protein would lack a large part of the ligand-binding domain and the AF-2 domain, but would contain the AF-1 domain, the DNA-binding domain and the nuclear localization signal of the wild-type ER. Leygue et al. (20) have identified ER transcripts in a wide range of human breast cancer samples, which are deleted from within exon 3 to within exon 7. This transcript was frequently detected in breast tumours, and in particular its expression was significantly correlated to tumours with very high levels of wild-type ER up to amino acid residue 232 and would then encode a further 18 novel amino acids (Fig. 1, Table 1). However, the protein lacks some of the

DNA-binding domain, all of the ligand-binding domain, and the AF-2 function.

7. Truncated ER mRNAs

The truncated ER-like transcripts (24, 25), which consist of various combinations of exons 1, 2 and 3 of the normal ER mRNA followed by sequences that are not found in the wild-type ER mRNA, were initially identified on Northern blots as abundantly expressed smaller-sized ER transcripts in some human breast cancer biopsy samples. This analysis identified them as abundant or more abundant than the wild-type transcript in some human breast cancer samples (24). Subsequently, several of the cognate cDNAs for these truncated transcripts were cloned and characterized, and found to contain authentic polyadenylation signals and poly A tails. The clone 24- and clone 5-truncated transcripts were found in only one breast tumour but the clone 4-truncated ER mRNA was found to be expressed in a wide range of breast tumours (25). Clone 5, however, consisted of exon 1 and 3 followed by ER unrelated sequences, and therefore is an example of a mixed exon-deleted and truncated transcript. Clone 4 consists of exons 1 and 2 of the wild-type ER mRNA followed by LINE-1 sequences (25). It could encode a protein of approximately 24 kDa, which would be identical to amino acid residues 1–214 of the wild-type human ER protein (25) (Fig. 1, Table 1) and thereafter encodes another six novel amino acids that are not found in the wild-type human ER. If the clone 4 mRNA were translated it would encode a protein that is identical to the A/B region and the first 'zinc finger' of the normal ER protein, but would be missing the second 'zinc finger', nuclear localization domains and the E domain of the normal ER protein (4). However, the protein had no transcriptional or dominant negative activity in transient transfection assays (25). Support for a role for this variant in human breast cancer progression comes from data that show that the relative level of expression of this variant is significantly elevated in breast tumours versus normal mammary gland (39) and that the relative level of expression of this variant is significantly elevated in breast tumours with characteristics of poor prognosis and endocrine resistance versus those with characteristics of good prognosis and endocrine sensitivity (41).

8. Point Mutations in the ER

Several point mutations have been identified in the human ER. The first of them was a G-to-C mutation (30–32), which was a silent polymorphism at nucleotide 261 (using the numbering presented in (64)). Although this is a silent polymorphism, the B-region variant allele (B′) of the ER has been correlated with decreased levels of oestrogen binding in human breast cancers (65), increased history of spontaneous abortion in women with ER-positive breast cancer (66), increased height in women (67) and possibly increased prevalence of hypertension (68).

A C-to-T transition at codon 157 in exon 2 of the human ER appears to be the cause of oestrogen resistance in a man (36). The mutation results in a premature stop codon so that a protein truncated within exon 2 would be formed, encoding only the A/B region and missing both zinc fingers of the DNA-binding domain as well as the entire hormone-binding domain. This is the first identified disease causing mutation in the human ER. Interestingly, this study demonstrated that disruption of the ER gene need not be lethal in humans and identified the importance of oestrogen in bone maturation and mineralization in men as well as women (36).

Point mutations have been identified in the ER in some breast cancers. A silent polymorphism (T-to-C) at serine 10 has been identified by at least two independent groups (25, 29). A leucine to proline substitution at amino acid residue 296 has been identified in two breast tumours (33); however, the functional significance of this is unknown. A C-to-G change that is a silent polymorphism at proline 325 (33) has also been observed. Karnik et al. (22) identified an A-to-T nucleotide change in one breast tumour, which would alter Glu 352 to Val as well as several silent polymorphisms (C-to-T in Gly 276; G-to-T in Lys 472; C-to-G at Ala 505; T-to-C at His 577). However, none of these was frequently observed and none correlated with tamoxifen sensitivity or resistance in this group of human breast tumours. The point mutation changing Gly 400 to Val that was introduced into the human ER cDNA, as a cloning artifact, was shown to alter the receptor's affinity for oestrogen under certain conditions (69), as well as to enhance the oestrogenic activity of 4-hydroxytamoxifen in stable ER transfectants of MDA-MB-231 human breast cancer cells (64). Moreover, the ER from an MCF-7 tumour line, which was stimulated by tamoxifen, contains a point mutation so that Asp 351 was changed to a Tyr residue (34). This mutant ER was subsequently shown to result in increased oestrogenicity of a tamoxifen analog (35).

9. Insertions in the ER

ER mRNAs containing inserted sequences have been identified in approximately 9% of human breast tumours (26). Three types of inserted sequences were identified: one in which a complete duplication of exon 6 was found, one in which a complete duplication of exons 3 and 4 was found, and one in which 69 novel nucleotides had been inserted between the exon 5 and 6 sequences of the normal ER mRNA. The functional significance of such alterations is as yet unclear. However, the exon 6-duplicated ER-like mRNA predicts a protein of 51 kDa identical to the wild-type ER but would be truncated in the mid-E domain. Deletion and site-directed mutagenesis data suggest that such a protein would not bind oestradiol (2, 4, 71–74). Further, an important dimerization interface and the ligand-dependent AF-2 activity would be missing in the protein predicted from the exon 6-duplicated ER-like mRNA. However, a weaker constitutively active dimerization domain present in the DNA-binding domain, as well as the constitutive nuclear localization signal present in

exon 4 of the wild-type ER (75) and the ligand-independent AF-1 activity in the A/B domain would still be present (5). Preliminary data suggest that the protein encoded by this transcript has no ability to bind oestradiol and has little, if any, transcriptional activity using a classical ERE reporter gene construct (D. Douglas and L. Murphy, unpublished observations).

The predicted protein from the exon 3 and 4-duplicated ER transcript is around 82 kDa (Fig. 2, Table 1). It is identical to the wild-type ER protein up to amino acid residue 366, followed by another 380 amino acid residues encoded by exons 3 to 8. Therefore the amino acid residues encoded by exons 3 and 4 are completely duplicated. This protein would contain the AF-1 domain located in the A/B region of the wild-type ER, as well as the DNA-binding and dimerization domains and the constitutive nuclear localization signal of the wild-type ER protein, but would then have a third zinc finger encoded by exon 3, another nuclear localization signal followed by the normal E-domain containing ligand binding, AF-2 and dimerization functions. The presence of the extra ER residues from exons 3 and 4 would probably result in an altered structure of the protein, which may affect several of its normal functions. Preliminary data suggest that the protein encoded by this transcript has reduced oestradiol-binding activity and reduced, but still detectable, ligand-activated transcriptional activity (D. Douglas and L. Murphy, unpublished observations).

The unique 69-bp insertion is in-frame and codes for 23 novel amino acids inserted between residues 412 and 413 of the normal ER protein (Fig. 2, Table 1). This would result in a protein of approximately 69 kDa. While all residues of the wild-type ER are present in this protein the inserted sequence may cause an alteration of the structure in the E domain of this protein, so that some alteration or disruption of function may occur. Preliminary data suggest the protein encoded by this transcript has reduced oestradiol-binding activity and little, if any, transcriptional activity of its own (D. Douglas and L. Murphy, unpublished observations).

Interestingly, the identification of an immunoreactive ER-like protein of 80 kDa was recently reported in an MCF-7 subclone, which was oestrogen independent with respect to growth (45). The transcript possibly corresponding to this protein appeared to contain a precise duplication of both exons 6 and 7 (Table 1, Fig. 2). Also, an abnormal ER-like transcript was cloned from T-47D_{CO} cells, which contained an insertion of approximately 130 nucleotides into exon 5 sequences (21). The inserted sequences displayed sequence similarity to the human *alu* family of repetitive sequences (21). The same group identified another mutant ER transcript in T-47D_{CO} cells, in which two T residues were inserted in exon 3 resulting in a frame shift, changing amino acid 250 from methionine to an isoleucine, followed by a stop codon (21). The predicted protein would be truncated just beyond the last cysteine of the second zinc finger, with no hinge or ligand-binding domains (Table 2). Although no DNA binding/gel retardation analysis for this predicted protein was observed, the protein displayed weak constitutive transcriptional activity, and higher concentrations had weak inhibitory activity when expressed together with the wild-type ER (76). In addition, some small insertions (1-3 nucleotides) have been described in the ER mRNA of some breast cancer biopsy samples (22, 29, 33) (Table 2). The frequency and significance of these are not known.

Conclusions and Unanswered Questions

There is a large amount of molecular evidence supporting the existence of variant and mutant ER proteins. While this evidence is derived mainly from characterization of mRNA species, data are now accumulating to suggest that the stable translation of ER variant mRNAs occurs at least in some human breast cancer tissues. This, in turn, suggests that any future examination of ER signal transduction and/or measurement of ER protein must take into account variant ER expression. The possible functions of variant ER proteins, either physiological or pathological, remain unclear, although correlative studies tend to support a role or roles for some ER variants in breast tumourigenesis and breast cancer progression. However, future speculation concerning these issues must take into account the presence of multiple ER variants in any one breast tissue sample, as well as the relative expression of each variant with respect to others, which can be altered in different groups of breast tumours, as discussed above. Furthermore, there are data that support the possibility that the pattern of ER variant expression can differ amongst different normal oestrogen target tissues (23), suggesting a possible role in the tissue-specific differences of ER signal transduction. These differences also dictate that analysis of putative function of any individual ER variant must also consider the cellular context as well as the promoter used to assess transcriptional function. This becomes increasingly important in the light of recent studies where novel oestrogen-responsive DNA sequences have been characterized, which remain quite distinct in structure–function activity and presumably mechanism from that classically determined using ERE sequences from the vitellogenin promoter (77–80). The recent cloning of a new ER, ER-beta (81, 82), with an overlapping but distinct pattern of tissue expression to the classical ER-alpha, also begs the question of whether the two ERs can interact and how the variant receptor forms may affect either or both signal transduction pathways.

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APPENDIX 2

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ESTROGEN RECEPTOR BETA: A REVIEW

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ABSTRACT

A single receptor, ER–α, was thought to mediate estrogen and anti-estrogen action in estrogen target tissues. Recently, a second estrogen receptor, known as ER–β, has been identified in several known (ovary, breast, bone) as well as "less conventional" (lung, heart, kidney) estrogen target tissues in human, mouse and rat. ER–β, which is also a member of the steroid/thyroid/retinoic acid receptor super-family, shares a similar structural and functional composition to ER–α and is able to activate the transcription of target genes through identical estrogen responsive elements. The observation of differential activation of ER–α and ER–β by anti-estrogens together with their ability to form hetero-dimers, suggests however that these two receptors might have different roles and that putative cross-talk of their signaling pathways might exist. Estrogen mechanism of action in any given tissue should therefore be re-evaluated. The purpose of this mini-review is to summarize the data published to date and to discuss the possible implications of the expression of ER–β in human breast cancer.

INTRODUCTION

Estrogens, that are known to regulate the growth and the development of reproductive female organs, also play key roles in other target tissues such bone, central nervous system and cardiovascular system. Estrogen effects were thought to be mainly mediated through a previously cloned and well characterized receptor, now referred to as estrogen receptor alpha (ER–α). ER–α, which belongs to the steroid-thyroid-retinoic acid receptor superfamily [1], was cloned in 1985 from a human breast tumor cell line cDNA library [2]. Like other members of this family, ER–α can be divided into several structural and functional domains (A-F) depicted in Figure 1 [3]. The A/B region of the receptor is involved in trans-activating function AF-1, whereas the C region contains the DNA-binding domain and the E region is implicated in hormone binding and another trans-activating function, AF-2. Upon ligand-binding, ER–α dissociates from a protein complex containing heat-shock proteins such as hsp 70 and hsp 90 to dimerize, and the resulting dimer binds to DNA at specific sequences called estrogen responsive elements (ERE) located upstream of the target gene [4]. Following interactions of the ER–α homo-dimer bound to the DNA and accessory co-activator proteins the transcription of such genes is eventually modified. Upon ligand binding, ER–α is also able to mediate the

transcription of AP1 regulated genes [5]. The ability of anti-estrogens such ICI-164,384, tamoxifen or raloxifene to bind ER–α and to modify its DNA-binding properties and its interactions with accessory proteins and ultimately its trans-activating activities, underlies their efficacy as endocrine therapies in breast cancer. In 1995, Kuiper et al. [6], isolated from a rat prostate cDNA library a 2.6 kb cDNA which encoded a molecule with strong sequence similarities to the DNA-binding domain (95%) and the hormone binding domain (60%) of ER–α (Figure 1). The discovery of this second estrogen receptor, called estrogen receptor beta (ER–β), led to the need to fully re-evaluate the molecular mechanisms of estrogen signal transduction in target tissues.

PRIMARY STRUCTURE AND VARIANT FORMS OF ER–β

Since the first report of the cloning of ER–β cDNA, that occurred more than two years ago, several groups have published (or submitted to Genbank) sequences of ER–β related molecules. However, the primary structure of ER–β still remains uncertain [7]. In order to understand the reasons behind the discrepancies observed, it is important to review the different cloning strategies used to identify ER–β related sequences.

Rat

Kuiper et al., looking for novel nuclear receptors, used degenerate primers, the sequences of which were based upon conserved DNA- and ligand–binding domains of nuclear receptors, to amplify rat prostate cDNAs [6]. They obtained a PCR product which when sequenced presented strong sequence similarities (65%) to the rat ER–α cDNA. Using this fragment as a probe, they isolated from a rat prostate cDNA library a 2.6 kb long cDNA which could encode a protein, initially called clone 29 protein, shown in Figure 2. This protein has strong sequence similarities with the ER–α DNA-binding and ligand-binding domains and the authors called this protein ER–β, to distinguish it from the previously identified estrogen receptor. Since then, several alternatively spliced forms of ER–β mRNA have been described and we will therefore refer to the protein encoded by clone 29 cDNA as rER–β1 (for rat ER beta 1). Because of the presence of an in-frame stop codon upstream of the coding sequence obtained, it was assumed that the 2.6 kb rat cDNA encoded the full length rER–β1 protein. The calculated molecular mass of the 485 amino acids encoded by this cDNA is 54.2 kDa. In vitro translated rER–β1 protein migrated as a doublet on SDS/PAGE with an apparent

molecular mass of 61 kDa. The presence of a doublet was explained by Kuiper et al. as the possible result of the use of two different initiating codons for protein synthesis, but no mention was made regarding the discrepancy between the calculated and the observed molecular mass. Subsequently, most other rat ER–β sequences were obtained using reverse-transcription (RT), and polymerase chain reaction (PCR) using primers spanning the initial published coding sequence. Therefore, most of them share the same initiating methionine codon and the last glutamine codon of rER–β1 (see Figure 2). In 1997, an inserted variant form of ER–β, referred to as rER–β2, was identified by Chu et al. after RT-PCR amplification of rat ovary cDNA [8]. The existence of such a variant was subsequently confirmed by other groups [9, 10]. This variant consists of an insertion of 54 bp between exon 5 and exon 6 of ER–β. One should note here that as underlined later in the text, the exon/intron structure of ER–β gene established by Enmark et al. [11] is similar to that previously shown for ER–α [12]. This 54 nucleotide insertion is in frame and therefore the inserted transcript will encode an extra 18 amino acids within the ligand-binding domain of the molecule (Figure 2). Variant forms of rER–β1 and rER–β2 deleted in exon 3 were also described and referred to as rER–β1Δ3 and rER–β2Δ3, respectively [10]. This in frame deletion of exon 3 would result in the elimination of the second zinc finger of the DNA-binding domain of the receptor (Figure 2). In 1998, Aldridge et al. submitted to Genbank (accession number AJ002602) an ER–β sequence obtained from a cDNA isolated by RT-PCR from rat prostate cDNA. These authors, in contrast to the previously mentioned studies, used an upper primer recognizing sequences upstream of the putative in frame upstream stop codon observed in rER–β1 cDNA. The 1650 bp long cDNA they obtained corresponds to the sequence between nucleotide 226 to 1874 of rER–β1 except for 6 differences. These differences consisted of an additional C residue between C319 and T320, an A instead of T at position 496, a G instead of C at position 729, a C instead of T at position 774, a C instead of T at position 1034, and a C instead of T at position 1794. The extra nucleotide observed between nucleotides 319 and 320 of rER–β1 sequence, alters the reading frame, suppresses the previously observed in frame upstream stop codon and results in this new ER–β sequence encoding an extra stretch of 64 amino acids upstream of the rER–β1 protein sequence (Figure 2). The resulting 549 amino acid long protein, rER–β1long, has a calculated molecular mass of 61.3 kDa. The nucleotide change at position 1034 does not affect the primary structure of the protein, but changes at other positions modify amino acid composition: glutamine, alanine, proline and proline are observed at position 27,

105, 120 and 450 instead of leucine, proline, serine and serine, respectively (See Figure 2). Interestingly, sequences published by Maruyama et al. [9] and Petersen et al. [10] also contained two of these amino-acids changes, at position 27 and 105. One should note that Genbank sequence AB012721 submitted by Maruyama et al. does not contain these modifications suggesting that these authors isolated two slightly different rER–β2 isoforms [9]. These slight amino-acid differences, that may result from the cloning strategies used or the tissue studied should be noted since previously, a single amino-acid modification within the first ER–α sequence published was later shown to have a functional effect. The alteration caused an apparent destabilization of this receptor and a modification of its affinity for 17-β-estradiol (E2) [13]. The question of whether or not rER–β amino-acid changes affect protein function remains to be addressed.

Mouse

Tremblay et al. used a combination of PCR and cDNA screening to obtain the first "full length" mouse ER–β, mER–β1 [14]. These authors using degenerate primers specific for the ligand-binding domain of rER–β1 amplified a 550 bp fragment from mouse ovarian cDNA, that had strong sequence similarities to the rER–β1 sequence. Using this fragment as a probe, they isolated from a mouse cDNA library 3 clones, the sequences of which started in the ligand-binding domain of the molecule and contained a poly-A tail. Using a downstream primer specific for their "new" 3' mouse sequence and an upstream primer spanning the first 21 bases of the 5'UTR of rER–β1 and ending with the putative initiator methionine codon, Tremblay et al. eventually obtained the sequence encoding the "full length" mER–β1 (Figure 3). This sequence was later confirmed by Petterson et al. [16] even though some nucleotide variations which modified amino acid sequence were observed. Alanine, threonine, asparagine, aspartic acid, histidine, arginine and glycine were observed at position 2, 97, 155, 333, 367, 400 and 466 instead of threonine, alanine, serine, glycine, proline, glycine and glutamic acid, respectively (Figure 3). The change at position 2 results directly from the sequence of the primer used by Petterson et al. to amplify the 5'-extremity of the cDNA, that encoded this amino acid change. Two sequences, recently submitted to Genbank, were both obtained by PCR amplification of mouse ovarian cDNA. They revealed that the N-terminal extremity of the mouse ER–β could be longer than previously shown. These two isoforms, mER–β1med and mER–β1long form, would encode a protein containing, compared to mER–β1, 45 and 64 additional N-terminal amino acids, respectively (Figure 3). The mER–β1med isoform would encode a protein starting at a methionine codon corresponding

to the methionine 20 of rER–β1long, whereas the mER–β1long form would encode a protein starting at a methionine corresponding to the first methionine of rER–β1long. Similar to what was observed in the rat, an insertional variant ER–β mRNA containing an extra-stretch of 54 nucleotides at the junction of exon 5 and 6 has been described in the mouse [15]. This cDNA could therefore encode a protein, mER–β2, identical to mER–β1, except for the presence of 18 additional amino acids within the ligand-binding domain (Figure 3). Variant mRNAs deleted in exon 5 (mER–β1Δ5), exon 6 (mER–β1Δ6), and exon 5+6 (mER–β1Δ5-6) have also been reported [15]. The deletion of exon 5 and of exon 6 separately leads to a shift in the open reading frame and the putative encoded proteins are therefore missing all of the C-terminal region of mER–β1 (Figure 3). In contrast, the double deletion "exon 5 + exon 6" does not change the coding reading frame and the encoded protein will be deleted in 91 amino acids within the ligand-binding domain/AF2 region (Figure 3). Because these variant forms of mER–β have been observed using targeted PCR (i.e performed using primers spanning only a small portion of the mER–β1 cDNA), no information is available to date to determine whether these deleted forms correspond to mER–β1, mER–β1med or mER–β1long (Figure 3). Similarly, the partial 3' sequence of mER–β2 and mER–β1Δ5-6 cDNA does not allow the unequivocal determination of the sequence of the C-terminal extremity of the putative proteins encoded by these variants (Figure 3).

Human

In 1996, Mosselman et al. used a similar approach to that of Kuiper et al. to screen a human testis cDNA library. They identified two cDNAs encoding a protein with strong sequence homology to hER–α [17]. Interestingly, the sequence similarity observed stopped in both clones at the exact junction between sequences encoded by exon 7 and exon 8 as determined by analogy to hER–α. This observation led the authors to conclude that their cDNAs represented incompletely spliced transcripts. They therefore used RACE PCR amplification of testis cDNA to obtain the 3'terminal extremity of their cDNA. The resulting cDNA could encode the protein depicted in Figure 4. As observed for the rat, the comparison of the amino acid sequence of hER–β with hER–α- showed a high conservation of the DNA-binding domain (96%) and of the ligand-binding domain (58%). However, the absence of an inframe stop codon upstream of the first Met suggested that the 5'coding extremity of the cDNA might be incomplete. In 1997, Enmark et al., using probes corresponding to regions encoding the N-terminal and hinge domains of rER–β1 isolated several partial

clones of hER–β from human ovarian and prostatic cDNA libraries [11]. The first 45 and the last 59 amino acids of the sequence were obtained by PCR amplification of human ovary cDNA, using primers derived from rER–β1. These clones were then joined by PCR amplification and restriction enzyme digestion. The sequence eventually obtained could encode a protein (hER–β1) almost identical to that encoded by the Mosselman cDNA, except for the presence of 8 additional N-terminal amino acids, homologous to rER–β1 sequences (Figure 4). In 1998, Ogawa et al. [19] screened a testis cDNA library with probes corresponding to the DNA-binding domain of rER–α, and identified a hER–β-like clone that contained extra 5' sequences in addition to the hER–β1 sequence. This sequence could encode a protein with N-terminal amino acid sequences highly similar to the N-terminal sequence of mER–β1med. Here again, PCR was necessary to obtain a full length cDNA. This amplification was performed using primers corresponding to the 5'-sequences of the new clone and to the previously published 3' extremity. The protein encoded by this "full length" cDNA is presented in Figure 4 (hER–β1long). This 530 amino acid protein has a calculated molecular mass of 59.2 kDa. In vitro translated hER–β1long migrated as a doublet with an apparent size of 60 and 57 kDa suggesting the use of two different initiating codons. More recently, Moore et al. screened a single stranded human testis cDNA library with biotinylated-hER–β1 oligonucleotides and isolated, in addition to hER–β1long, two full length variant ER–β cDNAs, which could encode hER–β2 and hER–β3 [20]. These ER–β isoforms are identical to hER–β1long protein, except that they differ in their C-terminal extremities (Figure 4). In particular, they do not contain the region encoded by exon 8 of the hER–β1 cDNA sequence. The 495 amino acid hER–β2 and the 513 amino acids hER–β3 proteins are missing a part of the ligand-binding domain of the hER–β1 molecule and are therefore smaller, with calculated molecular masses of 55.5 and 57.5 kDa, respectively. The hER–β2 isoform was also recently cloned by Ogawa et et al. [21]. These authors have named their isoform ERbetacx and it is identical to hER–β2. It is important to note that the suffix "–β2" describes in the human species a particular truncated variant whereas in the rodents it refers to an inserted variant. There is no evidence of an equivalent to the rodent inserted ER–β2 variant in human tissues [15]. Using PCR, Moore et al. [20] isolated partial cDNA sequences encoding hER–β4 and hER–β5 (Figure 4). These cDNAs share the sequence encoded by exon 7, but differ in their 3'extremity and do not contain exon 8 sequences. The putative proteins encoded by these variant cDNAs will therefore be missing a part of the ligand-binding domain of the hER–β1 molecule. One should note that because these cDNA

isoforms have been observed using RT-PCR amplification of only a limited region of the molecule, no information is available regarding the putative N-terminal sequence of the encoded proteins (i.e whether they correspond to hER–β1long or to hER–β1short). It is unclear at present if ER–β variants analogous to the C-terminally truncated hER–β2, hER–β3, hER–β4 and hER–β5 exist in the rodent. In 1997, using primers recognizing sequences in exons 1 and 8 of hER–β1, we have successfully amplified from human breast tissue RNA, a variant form of hER–β cDNA deleted in both exon 5 and exon 6 [22]. This in frame deleted cDNA could encode a hER–β like molecule, referred to as hER–β1Δ5-6 (Figure 4), which is deleted in 91 amino acids within the region containing the hormone binding domain and the trans-activating function 2 of the molecule. The existence of this variant as well as others, deleted in exon 5 (hER–β1Δ5) or exon 6 (hER–β1Δ6) was later confirmed [15, 18, Genbank AF074599]. These variant forms of hER–β mRNA have been observed using targeted PCR and only partial cDNA sequences are known. No information is available to date to determine whether these deleted forms correspond to hER–β1short or hER–β1long (Figure 4). Similarily, the C-terminal extremity of the putative hER–β1Δ5-6 protein remains to be determined (Figure 4).

It is interesting to note that amongst all ER–β sequences described to date, only four have been obtained by direct cDNA subcloning: rER–β1 [6], hER–β1long, hER–β2 and hER–β3 [20]. Since rER–β1 sequence was the first one published, it is considered the "wild-type" molecule. Most of the cDNAs isolated directly from cDNA libraries encoded partially truncated ER–β1 molecules that were presumed incomplete. The apparent high frequency of detection of such partial sequences raises the question of what molecule represents the major ER–β form in a given tissue. In other words, given the fact that hER–β1 and mER–β1 required the use of PCR to be isolated, should they be considered as the "wild-type" molecule in all tissues? Similarly, do longer and/or different variant forms, still unidentified, exist?

PRIMARY STRUCTURE AND CHROMOSOMAL LOCALIZATION OF THE ER–β GENE

As mentioned above, the primary structure of the ER–β gene was established by Enmark et al. for the mouse and the human [11]. Similar to the ER–α gene (Figure 5), the ER–β gene is composed of 8 exons. The

ER–β gene exon/intron structure appears conserved between the two species and corresponds with the exon/intron structure observed for ER–α. The ER–β gene however differs from hER–α gene by its length, 40kb versus >140kb. The chromosomal localization of the ER–β gene has been established for the human (14q22-24 [11) and the mouse (chromosome 12 [14]). Even though the structure of the rat ER–β gene has not been formally established to date, the existence of similar splice variants (such as rER–β2 or rER–β1Δ3) strongly suggests that the rat ER–β gene will share a similar structure to that observed in mouse and human.

FUNCTIONAL FEATURES

Most of the functional studies published so far, have been performed using the shorter molecules like rER–β1, rER–β2, mER–β1 or hER–β1short. As outlined above, these receptors may be missing the N-terminal extremity of the protein. This region, by homology to the N-terminal extremity of ER–α, could be involved in the trans-activating-function 1 of the protein. The AF1 domain is known to be partially responsible for the agonistic effect of anti-estrogens such as tamoxifen on ER–α. To our knowledge, no study investigating the putative functional differences between long and short ER–β forms has yet been published. The main functional features of the rat, the mouse and the human ER–β molecules have been summarized in Table 1.

Rat

In vitro and in vivo translated rER–β1 protein was shown to bind 17β-estradiol with Kd values around 0.4 nM [6, 9, 10]. Several other estrogenic substances such as diethylstilbestrol (DES), estriol or estrone are also able to bind rER–β1 [6, 10, 23]. In contrast, other nuclear receptor ligands such as testosterone, progesterone or corticosterone are unable to bind to rER–β1 [10, 23]. Kuiper et al. compared the relative affinity of numerous ligands for hER–α and rER–β1 by ligand competition experiments and concluded that rER–β1 had a ligand-binding spectrum which was overall similar to that of hER–α [23]. However, some compounds such 17α-estradiol or moxestrol had a higher relative affinity for hER–α than for rER–β1, whereas others, such as 4-hydroxy-tamoxifen (4-OH-Tam) and ICI164,384, had a higher relative affinity for hER–β. Comparing rER–β1 and hER–α ligand-binding domains (LBDs) recombinantly expressed in E.coli, Witkowska et al. found a close structural relationship between E2-bound rER–β LBD and hER–α LBD

complexes [24]. These authors also suggested that while no significant differences exist between the response of hER–α and rER–β1 LBDs to 4-OH-Tam and ICI164,384, some compounds such 16α-bromo-estradiol and genistein showed selective interactions with hER–α and rER–β1 LBDs, respectively. Gel mobility shift assays demonstrated the ability of rER–β1 homo-dimers to bind a consensus ERE, even though the binding affinity observed was lower than that seen for rER–α homo-dimers [10]. Co-transfection experiments using an ERE-driven reporter gene and a rER–β1 expression vector revealed a stimulatory effect of 17–β-estradiol [3, 9, 10, 25]. Since these assays were performed in several different cell systems (CHO, Hela, Cos and 293S cells), this effect appears to be cell type independent. Kuiper et al. also showed that tamoxifen acts as an antagonist of E2 action in CHO cells [6]. Interestingly, the activity of rER–β1 via AP1-driven reporter genes differs significantly to that observed for ER–α. Paech et al. [25] demonstrated that whereas estrogens such E2 or DES stimulated the transcription of such genes when bound to ER–α, they inhibited transcription when associated with rER–β1. Moreover, while anti-estrogens such tamoxifen, ICI164,384 or raloxifene do not activate ER–α, they induce the transcription of AP1-regulated genes when bound to rER–β1.

The rER–β1 was shown to form hetero-dimers with rER–α and rER–β2 [9, 10], and the formation of rER–β1/rER–α hetero-dimers was favoured over rER–β1/rER–β1 and rER–α/rER–α homo-dimer formation in the presence of E2 [10]. The ability of ER–α and ER–β to hetero-dimerize adds an important layer of complexity to the mechanisms of estrogen signal transduction. Maruyama et al. showed that, in contrast to rER–β1, rER–β2 was unable to bind E2 [9]. However, Petersen et al. found that rER–β2 can bind E2 but with a markedly reduced affinity (Kd value of 5.1 nM) [10]. Both of these studies showed that rER–β2 was able to bind a consensus ERE. Whereas Petersen et al. showed that in the presence of 10nM E2, rER–β2 could activate the transcription of an ERE-reporter gene, Maruyama et al. found no activation of an ERE reporter gene under similar conditions. Using co-transfection experiments, Maruyama et al. observed a dominant negative effect of rER–β2 on estrogen activation of an ERE reporter gene by rER–β1, whereas Petersen et al. concluded that the relative expression of the two receptors modulates the effective dose of E2 required to obtain the maximal response. The discrepancies observed between these two studies may result from the use of different rER–β2 protein preparations (i.e fusion protein expressed in E.coli versus protein extract of rER–β2 transfected into 293T cells, for Maruyama's and Petersen's studies, respectively), and/or the amino-acid differences mentioned earlier in the text. Differences in transactivation studies may also be

attributed to the different cell systems used, Cos cells and 293S cells for Maruyama's and Petersen's study, respectively. The deletion of the amino-acids encoded by the exon 3, which causes the deletion of the second zinc finger, results in rER–β1Δ3 and rER–β2Δ3 both being unable to bind a consensus ERE [10]. The affinity for E2 of rER–β1Δ3 and rER–β2Δ3 does not differ from that observed for rER–β1 and rER–β2, respectively [10].

Mouse

As for rER–β1, mER–β1 has been shown to bind E2 with a Kd value of 0.5 nM [14]. It has also been established that mER–β1 is able to bind in vitro to a consensus ERE sequence [14, 16]. Ligands such E2, 4OHTam or ICI182,780 do not have any effect on mER–β1/ERE binding, even though different experimental conditions (i.e changing the pre-incubation temperatures) may highlight a role of E2 on the formation of mER–β1/DNA complex [14]. Co-transfection experiments performed using an ERE-driven reporter gene and mER–β1 expression constructs into Cos and Hela cells demonstrated the activation of mER-β1 by E2 (10 nM). This activation activity was antagonized by 4-OH-Tam and ICI182,780 [14, 16]. The co-transfection of the steroid receptor co-activator SRC-1, previously shown to interact with and to activate hER–α [26, 27], increased both ligand-dependent and -independent mER–β1 activation [14]. Tremblay et al. showed that 4-OH-Tam and ICI182,780 were able to interfere with SRC-1 mediated ligand independent activation of mER–β1 [14]. Similar to previous studies with ER–α [28, 29], Tremblay et al. also demonstrated that the activation of mER–β1 could be increased via activation of the Ras-Raf-1-MAPK kinase-MAPK pathway [14]. The complete disappearance of this potentiation in the presence of ICI182,780 but not of 4-OH-Tam led the authors to conclude that the Ras mediated effects are likely mediated by a putative AF-1 domain located within the N-terminal region of mER–β1. The target of MAPK phosphorylation was identified as the Serine residue located at position 60 of mER–β1 sequence [14]. mER–β1, like rER–β1, can form homo-dimers or hetero-dimers with mouse and human ER–α [16].

mER–β2, like rER–β2, is able to bind to an ERE consensus sequence in vitro, does not bind E2 in ligand-binding assays but inhibits the trans-activation of mER–β1 on classical ERE-tk-CAT reporter genes (Lu, B. personal communication). No data have been published so far regarding the putative functions of mER–β1med, mER–β1long, mER–β1Δ5, mER–β1Δ6 or mER–β1Δ5-6.

Human

Similar to hER–α, hER–β1short binds consensus ERE sequences in vitro [30]. Using various experimental conditions (different pre-incubation temperatures), Pace et al. showed that whereas ER–α and hER–β1short bind DNA in a similar manner in the presence of E2 or tamoxifen, their DNA-binding capabilities were slightly different in the absence of ligand or in the presence of the anti-estrogen ICI182,780 [30]. Transfection experiments performed using different cell systems (CHO, Cos 7 or Hela cells), revealed that, as observed in rodents, hER–β1short also activates the transcription of an ERE-driven reporter gene in the presence of E2 [17, 31, 32]. Moreover, as previously demonstrated for hER–α, anti-estrogens such tamoxifen, raloxifene, EM-800 or ICI164,384 are able to suppress the estrogen activation of hER–β1short [17, 32, 33]. Similar to the rER–β1 and in contrast to hER–α, hER–β1short activates the transcription of AP1-regulated genes when bound to anti-estrogens such raloxifene, ICI164,384 or tamoxifen [25]. These effects were observed in several different cell types such as human endometrial carcinoma Ishikawa cells or human epithelial breast cancer MCF-7 cells. Barkhem et al. also showed that ER–α and hER–β1short respond differently to some other synthetic estrogen agonists or antagonists [31]. For example, 17α-ethynyl,17β-estradiol selectively potentiates ER–α whereas 16β,17α-epiestriol has selective agonist properties via hER–β1short. Similarly, while agonistic effects of tamoxifen and raloxifene are observed in several cell systems (Cos 7, Saos, HG63 and Hela cells) using ERE-CAT reporter constructions via hER–α, no effect can be observed using hER–β1short [32].

Taken together, all of these observations suggest that differences in estrogen and anti-estrogen action can occur when the ligands are bound to hER–α or hER–β1, which are promoter and possibly cell type specific. Like rodent ER–β proteins, hER–β1short can form homo-dimers as well as hetero-dimerize with hER–α [30, 34]. Hetero-dimerization of the two receptors occurs independently of ligand through the DNA-binding domain of hER–α [30]. Recently, Ogawa et al. demonstrated that ERbetacx, also known as hER–β2, was not able to bind DNA. However, this is contrast to the observation of Moore et al. [20]. Erbetacx was unable to bind E2 and in a transient transfection experiment did not transactivate an ERE-reporter gene [21]. Ogawa et al. also showed that hER–β2 was able to form hetero-dimers with hER–α as well as with hER–β1long, although a preference for hetero-dimerization with hER–α was noted. Interestingly, hER–β2 can act as a dominant negative inhibitor of hER–α induced but not hER–β1long

induced transcription. All possible combinations of homo- and hetero-dimers have been observed between hER–β1long, hER–β2, hER–β3 and hER–α [19, 20]. No function has yet been attributed to the truncated variants hER–β3, hER–β4, hER–β5, hER–β1Δ5, hER–β1Δ6 or hER–β1Δ5-6. As described previously for hER–α by Ince et al. [35], Ogawa et al. demonstrated that a C-terminally truncated hER–β1long construct could act as a dominant negative inhibitor of hER–α and hERb1long transactivation of an ERE-driven reporter gene [36]. Similarly, a naturally occurring truncated variant of hER–α, encoded by an exon-7 deleted variant mRNA, has been shown to act as a dominant negative inhibitor of wild-type hER–α action [37]. In the light of such data plus those accumulating regarding hER–β2 [21], it could be suggested that hER–β3, hER–β4 and hER–β5 (truncated in the amino acids encoded by exon 8 of the wild-type hER–β1 cDNA) may share similar inhibitory functions on hER–α transcriptional activity. As for hER–β1Δ5, one could speculate, by analogy to what has been described for the variant encoded by an exon-5-deleted ER–α mRNA, that this isoform may have constitutive transcriptional activity [38].

TISSUE EXPRESSION

The possible expression of all ER–β isoforms mentioned above limits the interpretation of ER–β tissue expression studies published to date. In situ detection of ER–β protein or mRNA is performed using specific targeted probes that cover only limited regions of the molecule and would fail to give any information regarding what exact isoform is detected. For example, antibodies raised against the N-terminal region of hER–β1short may allow the detection of hER–β1long, hER–β1short, hER–β2, hER–β3, hER–β4, hER–β5, hER–β1Δ5, hER–β1Δ6 and hER–β1Δ5-6 isoforms. Inversely, an antibody raised against the C-terminal extremity of hER–β1short molecule may also recognize hER–β1long and hER–β1Δ5-6 and may or may not recognize hER–β2, hER–β3, hER–β4 or hER–β5 depending on the exact localization of the epitopes recognized. Moreover, we cannot exclude the existence of other still unknown isoforms that may also be recognized by such antibodies. The difficulty of interpretation of immunocytochemical results obtained using C-terminal and N-terminal antibodies able to differentially recognize several truncated variants was previously highlighted for hER–α [39] and has recently being reported by Rosenfeld et al. for mER–β1 immuno-detection [40]. Similar limitations exist regarding the in situ detection of ER–β related mRNAs. The previous demonstration of different sized ER–β like transcripts observed in testis or in ovary by Northern

blot analysis [11, 14] supports the expression of several different ER–β related mRNAs, an observation which should not be neglected when interpreting in situ hybridization results. Similarly, results obtained using other techniques such as Western blot, RT-PCR, RNase protection assay, or Northern blot, performed on homogenized tissue extracts, not only fail to give any information regarding the cell-specific pattern of expression of the molecules detected, but also are limited due to the heterogeneity of the detectable molecules. For example, hER–β2 is expected to migrate on a SDS-PAGE gel with an apparent mass of 55.4 kDa and cannot be discriminated from hER–β1short (54.2 kDa) by Western blot analysis. Similarly, even though the detection of variant isoforms by RT-PCR, performed using primers recognizing a specific variant, will provide the proof of the existence of a molecule with this particular sequence, no information about other regions of this molecule will be obtained. For example detecting ER–β4 using a primer in exon 7 and another primer specific for hER–β4 3' sequences will not answer the question: Does this isoform correspond to hER-β1short, hER–β1long or any other variant such as hER–β1Δ5-6? Table 4, Table 5 and Table 6 summarize the data reporting the detection of ER–β related molecules in rat, mouse and human tissues, respectively. These tables should be read keeping in mind that the method of detection of ER–β1 might also include several other isoforms, and that in none of these studies have probes been used which would establish whether the short, the medium or the long forms of ER–β1 were detected. Moreover the detection of other isoforms refers only to the detection of a region of the molecule recognized by the probe used. The particular mode of detection, i.e RT-PCR, in situ hybridization, Northern blot and RNase protection assay or Western blot and immunohistochemistry is indicated. The sensitivity of the technique used to detect ER–β related molecules together with the probe used might indeed be responsible for some discrepancies between studies. The ER–β gene is apparently expressed in a significant number of tissues. Some of these tissues, like breast, uterus or ovary, are known to depend on estrogens for their growth and their differentiation. On the other hand, some of the tissues expressing ER–β related molecules, such as spleen, lung or kidney are not usually considered as "conventional" targets for estrogen action. The level of expression of a particular ER–β isoform may vary significantly from one tissue to another [6, 10, 23, 53]. For example, studies performed on rodent tissues using in situ hybridization and RNase protection assay reveal a much higher expression of rER–β1 and mER–β1 mRNA in the prostate and ovary than in the epididymis, the testis or the uterus [6, 23]. Similarly, PCR analysis of hER–β4 expression revealed a strong signal in human testis but a weak one in spleen or

mammary gland [20]. For a given tissue, the level of expression of a particular isoform may also differ from one species to another. For example, the expression of ER–β1 is high in rat prostate, but apparently low in human prostate [11, 53]. Moreover, the expression of some isoforms may also be species specific: no equivalent of the inserted variant rER–β2 or mER–β2, strongly expressed in multiple rodent tissues, can be detected in the corresponding human tissues [15]. This last observation, which suggests that different mechanisms of estrogen signal transduction may exist between rodent and human suggests caution in the interpretation of data where rodent models have been used to study human estrogen dependent diseases, such as breast cancer. Within a single tissue, the cells expressing ER–α and ER–β may differ. For example Prins et al. showed that rat cells expressing rER–β1 were the prostatic epithelial cells whereas rER–α is expressed within the prostatic stroma [42, 60]. Similarly, hER–β is detected in developing spermatids but not in Leydig cells of human testis, whereas hER–α is not expressed in spermatids but is expressed in Leydig cells in human testis [11, 61].

To date, the biological significance of the expression of multiple ER–β isoforms remains to be established for each tissue. Amongst other effects, estrogen was shown to prevent bone lost resulting from osteoporosis [62], to exert a protective effect against atherosclerosis [63], to prevent the neuronal loss associated with Alzheimer's disease [64, 65] and to increase the risk of breast tumorigenesis [66, 67]. The detection of ER–β gene expression in bone, vascular system, brain and mammary gland therefore suggests a possible role of ER–β isoforms in estrogen signal transduction in these tissues. Implications of such expression have been discussed in several articles [52, 68- 73]. The rest of this article will focus on the possible role of ER–β expression in human breast cancer.

hER–β AND BREAST CANCER

Estrogens, that regulate the normal growth of human mammary tissue, are also involved in the progression of mammary cancer [66]. Before the discovery of ER–β, the effects of estrogens were thought to be mainly mediated through ER–α. The presence of variant ER–α proteins, that would be encoded by the numerous ER–α variant mRNAs detected in breast tissue, has led to the hypothesis that they could interfere with wild-type hER–α signaling pathways and therefore contribute to the apparent loss of estrogen sensitivity observed

during breast tumorigenesis [74]. It has been suggested that estrogen responsiveness may be influenced by a certain balance between all ER–α isoforms. A change in this balance, resulting in a change in estrogen responsiveness and sensitivity, may underly breast tumorigenesis and breast tumor progression [75]. Indeed, several groups have reported changes in the relative expression of particular hER–α variants during breast tumorigenesis. For example hER-αΔ5, hER-αΔ7 and clone 4 mRNAs (a truncated hER–α variant mRNA) were found more highly expressed in tumor tissue than in normal breast tissue [76, 77]. Similarly a higher expression of clone 4 correlated with parameters of poor prognosis and endocrine insensivity [78]. A higher expression of hER-αΔ5 was also detected in ER-/PR+ than in ER+/PR+ tumors [38], whereas ER+/PR+ tumors expressed a lower level of hER-αΔ7 than ER+/PR- tumors [37]. These data support the observation that a change in hER–α isoforms balance occurs during breast tumor progression. Several studies have now described the expression of ER–β related mRNAs in normal and neoplastic human breast tissues (see Table 4, and references [11, 15, 18, 58, 79]). The functional features of some of these ER–β isoforms, underlined above in the text, suggest that ER–β proteins not only participate in the mediation of E2 effects in breast but may also modulate hER–α signal transduction. Such modulation may result from the competition for ligand, for DNA-binding, for co-activators and/or via numerous possible hetero-dimerization combinations. One could hypothesize that the number of possible combinations may also be increased by yet to be demonstrated ER–α variants/ER–β variants hetero-dimerization. It is reasonable to assume that estrogen action in breast tissue will depend therefore on the balance between all hER–α and hER–β isoforms. We first reported in 1997 the presence of hER–βΔ5-6 variants in breast tissue and suggested that hER–β variants may also be involved in the mechanisms underlying tumor progression [22]. The increasing number of hER–β isoforms identified to date stresses the need to determine expression levels and possible changes in hER–α/hER–β isoforms in both normal and neoplastic human breast tissue. Using a semi-quantitative RT-PCR approach to investigate the relative proportion of ER–α and ER–β in matched human normal and tumor breast samples, we have recently reported that changes occur in the balance between the two receptor [79]. We showed that in a cohort of ER+ breast tumors, a higher ER–α/ER–β ratio was observed in the tumor compared with that of the matched adjacent normal tissue component. The increased ER–α/ER–β ratio resulted primarily from an increase in ER–α mRNA expression in conjunction with in most cases, a decrease of ER–β mRNA levels in the tumor compared to the normal tissue. These data therefore suggest that the balance between the two

receptors is modified during breast tumorigenesis. Interestingly, a change in ER–α and ER–β signaling pathways, as determined by the relative expression of ER–α and ER–β, occurs during ovarian tumorigenesis [56]. Brandenberger et al. demonstrated that the expression of ER–α was equal or higher in ovarian cancer than in normal ovary whereas in contrast, ER–β expression was lowered in tumor tissue. A change in ER–β expression has also been described in chemically transformed human breast epithelial cells [80]. In contrast to the two studies mentioned above, these authors described an increase in ER–β mRNA levels paralleling transformation. One should note that even though the ER–β change does not go in the same direction, i.e decreasing from normal to neoplastic, the modification of expression also describes a shift in the balance between the two receptors.

CONCLUSION

Since its cloning in 1995, ER–β has been the object of an extensive research effort. The increasing knowledge of the functional features of each ER–β isoform, together with its relative expression compared to other ER isoforms will hopefully soon allow a better understanding of its mechanism of action, alone or in the presence of other ER–α or ER–β molecules. However, the system is obviously complex, with many possible players as outlined in this review. Important questions still remain to be addressed: How many isoforms still remain to be identified? Do specific EREs exist for the different homo- and hetero-dimer combinations? What technique could be used to identify with certainty the isoforms observed? What are the genes specifically regulated by the the different homo- and hetero-dimer combinations? What are the actions of the different ligands on the different hetero-dimers? Do differences in the relative expression of various ER–β or ER–α variant isoforms underly well known species differences with respect to antiestrogen action, and/or the development of antiestrogen resistance in human breast cancer?

Together with other exciting avenues of research with respect to estrogen action, i.e specific ER mediators (SERM) and coactivators/corepressors [81, 82], the existence of two ERs (ER–α or ER–β) and their multiple variant isoforms provides an incredibly exciting and challenging research environment, the results of which will impact significantly in many areas of human health.

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TABLE LEGENDS

Table 1

Functional features of rat, mouse and human ER–β isoforms. For each isoform, the possibility to bind E2 (+ or -), to recognize an ERE in a gel shift assay (+ or -), or to activate an ERE-reporter gene (+ or -) in co-transfection assays is indicated. + - refers to studies with discrepant results. The effects of estrogens or antiestrogens on AP1-driven reporter genes are also indicated. The molecules shown to form hetero-dimers with each isoform are cited. Ref: references.

Table 2

Tissue detection of rat ER–β isoforms. For each tissue, the isoform detected as well as the mode of detection. ER–β mRNAs were detected using RNase protection assay (R), in situ hybridization (I), Northern blot (N) or RT-PCR (P). ER–β proteins were detected using immuno-histochemistry (H) or Western blot (W).

Table 3

Tissue detection of mouse ER–β isoforms. For each tissue, the isoform detected as well as the mode of detection. ER–β mRNAs were detected using RNase protection assay (R), in situ hybridization (I), Northern blot (N) or RT-PCR (P). ER–β proteins were detected using immuno-histochemistry (H) or Western blot (W).

Table 4

Tissue detection of human ER–β isoforms. For each tissue, the isoform detected as well as the mode of detection. ER–β mRNAs were detected using RNase protection assay (R), in situ hybridization (I), Northern blot (N) or RT-PCR (P). ER–β proteins were detected using immuno-histochemistry (H) or Western blot (W). NB: two studies were performed on monkey tissues [57, 59]

FIGURE LEGENDS

Figure 1

Structural and functional domains of rat estrogen receptor alpha (rER–α) and beta (rER–β). Region A/B of the receptor is implicated in trans-activating function (AF-1). The DNA-binding domain is located in the C region. Region E is involved in ligand-binding and another trans-activating function (AF-2). For each receptor, the length (aa), the calculated mass (kDa) and the amino acid positions of the different domains are given. Percentage amino acid identity in each domain is indicated.

Figure 2

Rat ER–β isoforms. All ER–β isoforms are aligned. Amino acid positions of the different structural domains are indicated for rER–β1 (Genbank RNU57439, AF042058). White boxes indicate identity of amino acid between sequences. Discrepancies in all published amino acid sequences at position 27, 105, 120, and 460 of rER–β1 are indicated by asterisks. The eighteen amino acid insertion within the LBD/AF2 domain observed in rER–β2 ([8], Genbank RNAJ2603, AF42059, AB012721) is indicated by a gray box. rER–β2Δ3 (Genbank AF42061) and rER–β1Δ3 (Genbank AF42060) are missing the second zinc finger of the DNA-binding domain encoded by exon 3. rER–β1long (Genbank RNAJ2602) contains 64 additional N-terminal amino acids. For each receptor, the length (aa) and the calculated mass (kDa) are given.

Figure 3

Mouse ER–β isoforms. All ER–β isoforms are aligned. Amino acid positions of the different structural domains are indicated for mER–β1 (Genbank MMU81451, MMAJ220). White boxes indicate identity of amino acid between sequences. Discrepancies in all published amino acid sequences at positions 2, 97, 155, 333, 367, 400 and 466 of mER–β1 are indicated by asterisks. The eighteen amino acid insertion observed in mER–β2 [15] is depicted by a gray box. mER–β1Δ5 and mER-β1Δ6 [15] are truncated and contain different C-terminal amino acids (black boxes). mER–β1Δ5-6 is missing 91 amino acid within the LBD/AF2 domain [15]. mER–β1med (Genbank AF063853) and mER–β1long (Genbank AF067422) contain 45 and 64 additional N-terminal amino acids, respectively. For each receptor, the length (aa) and the calculated mass

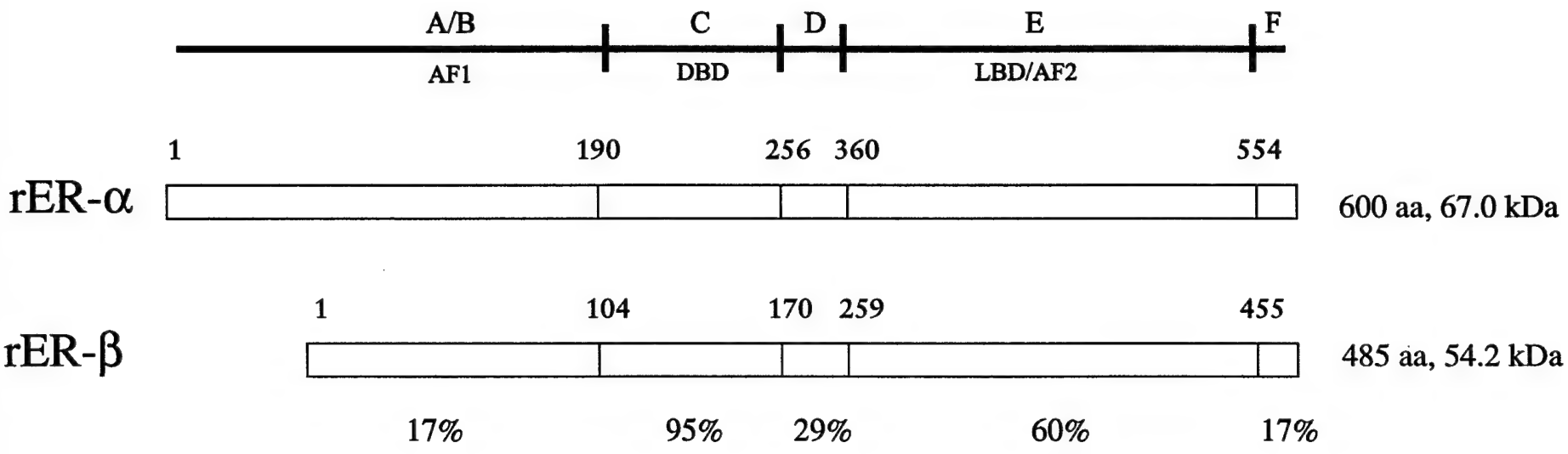
(kDa) when known or corresponding to the short (S), the medium (M) or the long (L) forms of the putative proteins are given. Broken boxes and question marks indicate that flanking amino acid sequences are unknown.

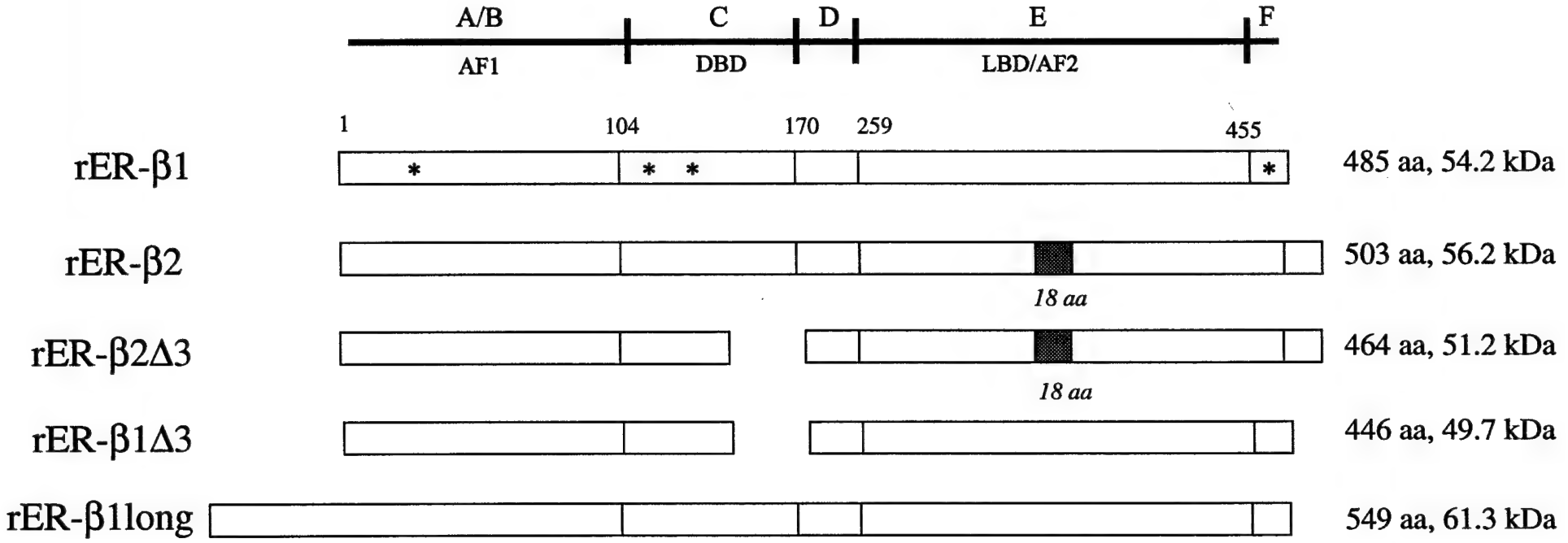
Figure 4

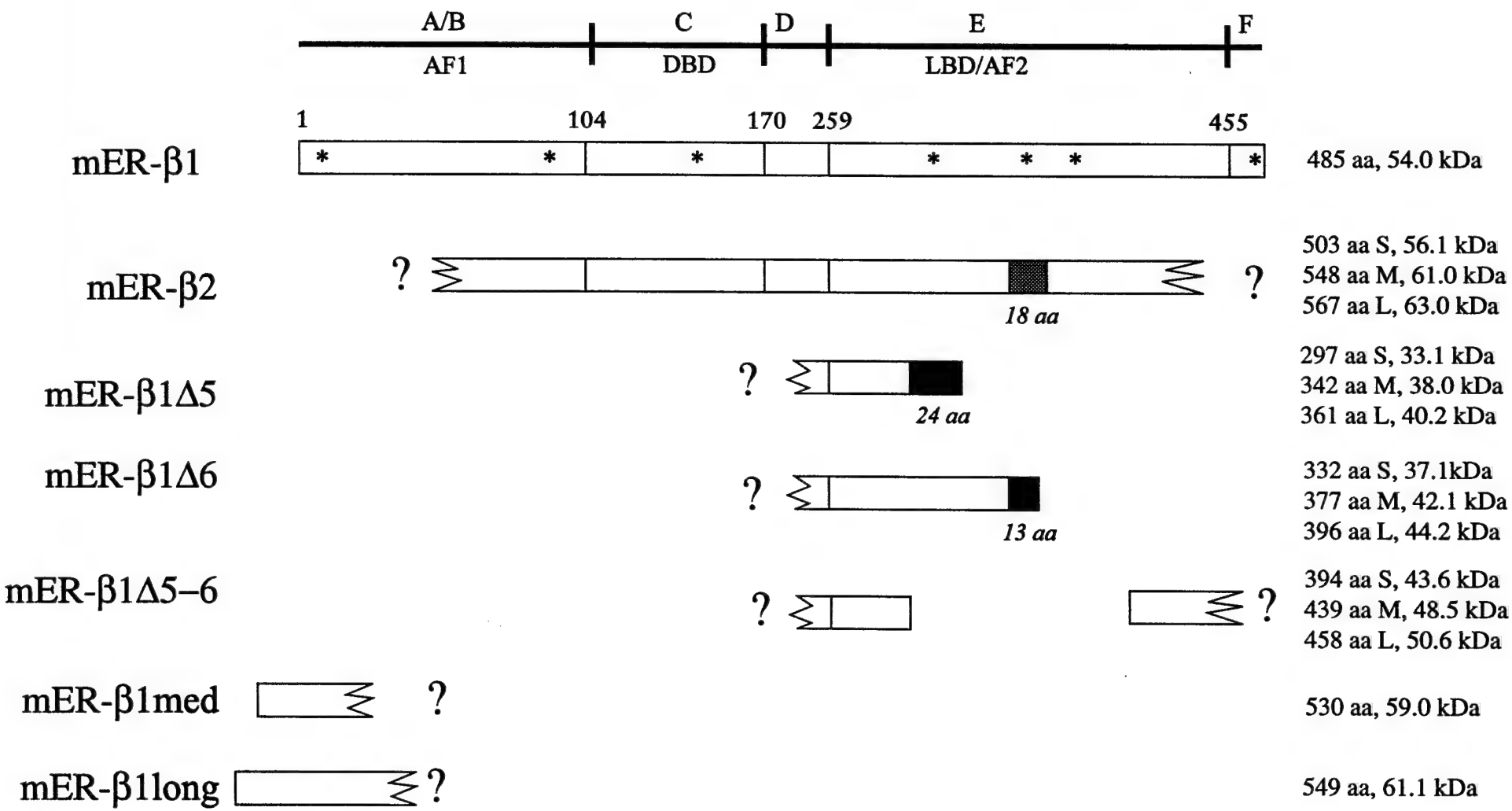
Human ER–β isoforms. All ER–β isoforms are aligned. White boxes indicate identity of amino acid between sequences. Amino acid positions of the different structural domains are indicated for hER–β1short [11] that contains 8 extra N-terminal amino acids compared to the first hER–β described [17]. hER–β1long (AF051427) contains 45 additional N-terminal amino acids. hER–βΔ5 [15, 18], hER-βΔ6 [15], hER–β2 (Genbank AF051428, AB006589cx), hER–β3 (Genbank AF060555), hER–β4 (Genbank AF061054), hER–β5 (Genbank AF061055) are truncated and contain different C-terminal amino acids (black boxes). hER–βΔ5-6 ([15], Genbank AF074599) is missing 91 amino acids within the LBD/AF2 domain. For each receptor, the length (aa) and the calculated mass (kDa) when known or corresponding to the short (S) or the long (L) forms of the putative proteins are given. Broken boxes and question marks indicate that flanking amino acid sequences are unknown.

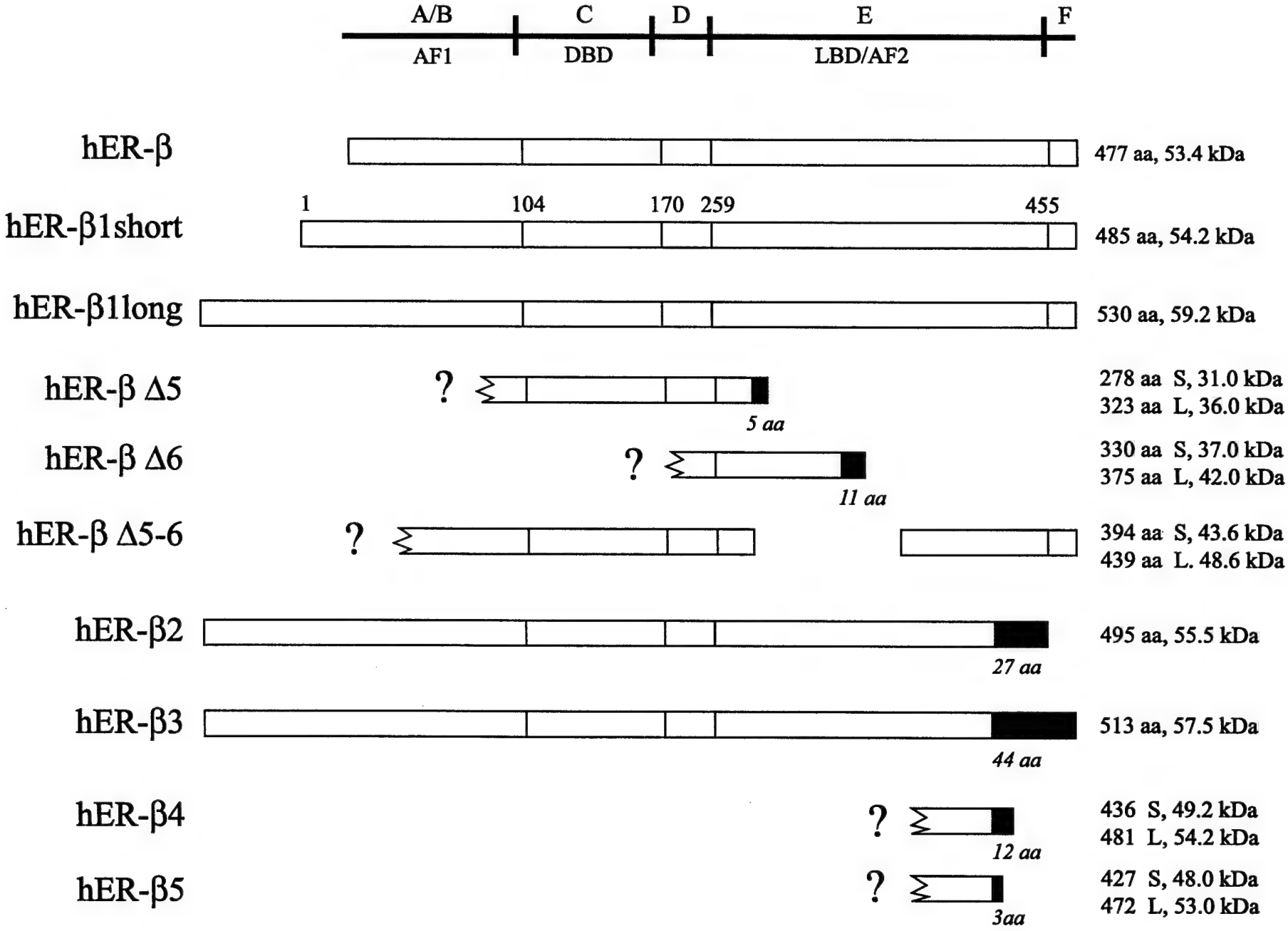
Figure 5

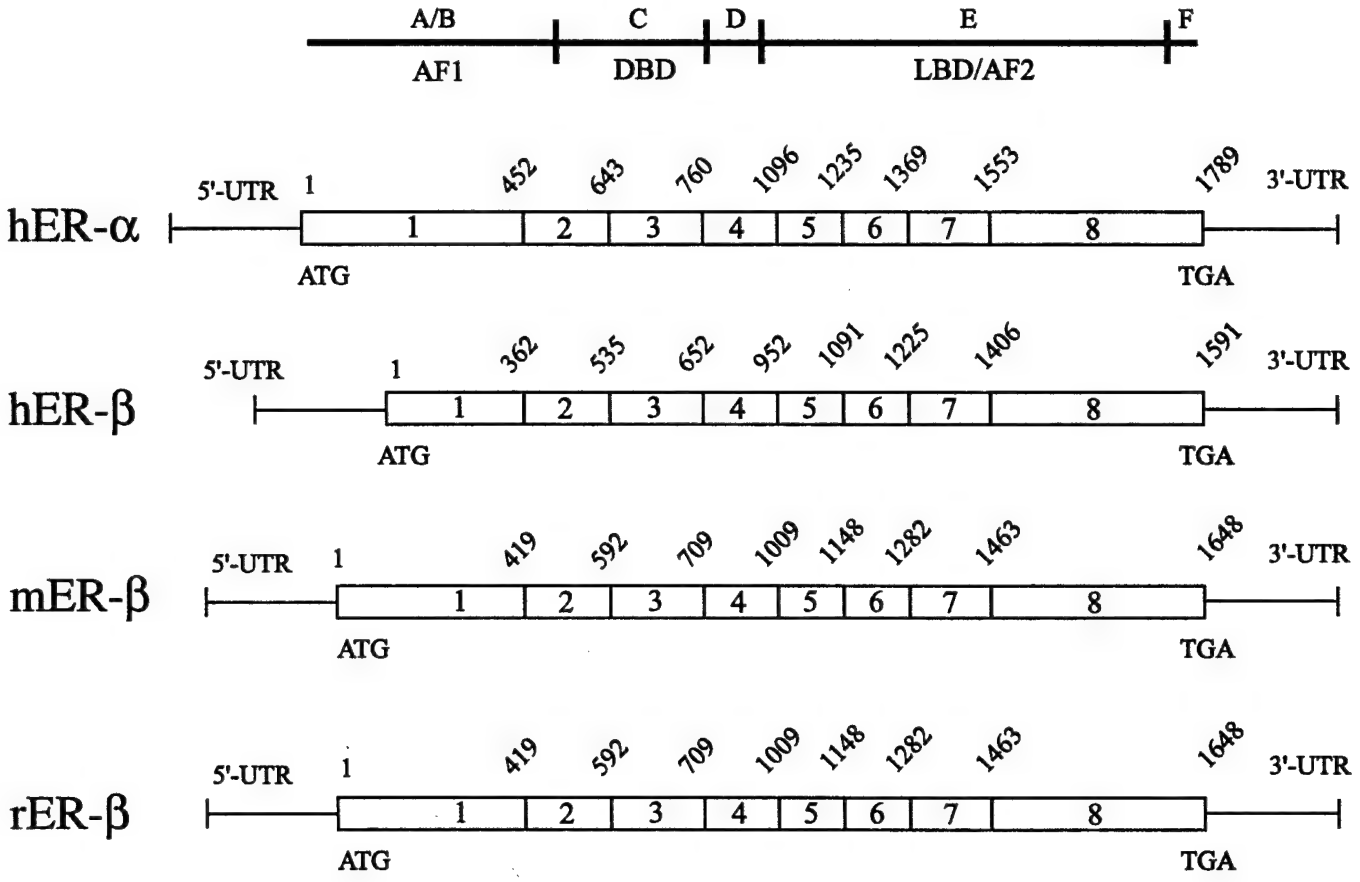
Exonic structure of human ER–α and ER–β. The exon structure of hER–α, hER–β, mER–β and rER–β cDNA are schematically depicted. The nucleotides are numbered starting at 1 for the ATG corresponding to the first methionine codon of the longest ER–β1 transcript observed for each species, i.e hER–β1long (Genbank AF051427), mER–β1long (Genbank AF067422) and rER–β1long (Genbank RNAJ2602).


A/B
C
D
E
F
AF1
DBD
LBD/AF2
1
190
256
360
554
rER-α
600 aa, 67.0 kDa
1
104
170
259
455
rER-β
485 aa, 54.2 kDa
17%
95%
29%
60%
17%


A/B
C
D
E
F
AF1
DBD
LBD/AF2
1
104
170
259
455
rER-β1
485 aa, 54.2 kDa
rER-β2
503 aa, 56.2 kDa
18 aa
rER-β2Δ3
464 aa, 51.2 kDa
18 aa
rER-β1Δ3
446 aa, 49.7 kDa
rER-β1long
549 aa, 61.3 kDa


A/B
C
D
E
F
AF1
DBD
LBD/AF2
1
104
170
259
455
mER-β1
485 aa, 54.0 kDa
mER-β2
503 aa S, 56.1 kDa
548 aa M, 61.0 kDa
567 aa L, 63.0 kDa
18 aa
mER-β1Δ5
297 aa S, 33.1 kDa
342 aa M, 38.0 kDa
361 aa L, 40.2 kDa
24 aa
mER-β1Δ6
332 aa S, 37.1kDa
377 aa M, 42.1 kDa
396 aa L, 44.2 kDa
13 aa
mER-β1Δ5–6
394 aa S, 43.6 kDa
439 aa M, 48.5 kDa
458 aa L, 50.6 kDa
mER-β1med
530 aa, 59.0 kDa
mER-β1long
549 aa, 61.1 kDa


A/B
C
D
E
F
AF1
DBD
LBD/AF2
hER-β
477 aa, 53.4 kDa
1
104
170
259
455
hER-β1short
485 aa, 54.2 kDa
hER-β11long
530 aa, 59.2 kDa
hER-β Δ5
?
5 aa
278 aa S, 31.0 kDa
323 aa L, 36.0 kDa
hER-β Δ6
?
11 aa
330 aa S, 37.0 kDa
375 aa L, 42.0 kDa
hER-β Δ5-6
?
394 aa S, 43.6 kDa
439 aa L. 48.6 kDa
hER-β2
27 aa
495 aa, 55.5 kDa
hER-β3
44 aa
513 aa, 57.5 kDa
hER-β4
?
12 aa
436 S, 49.2 kDa
481 L, 54.2 kDa
hER-β5
?
3aa
427 S, 48.0 kDa
472 L, 53.0 kDa


A/B
C
D
E
F
AF1
DBD
LBD/AF2
hER-α
5'-UTR
1
452
643
760
1096
1235
1369
1553
1789
3'-UTR
1
2
3
4
5
6
7
8
ATG
TGA
hER-β
5'-UTR
1
362
535
652
952
1091
1225
1406
1591
3'-UTR
1
2
3
4
5
6
7
8
ATG
TGA
mER-β
5'-UTR
1
419
592
709
1009
1148
1282
1463
1648
3'-UTR
1
2
3
4
5
6
7
8
ATG
TGA
rER-β
5'-UTR
1
419
592
709
1009
1148
1282
1463
1648
3'-UTR
1
2
3
4
5
6
7
8
ATG
TGA

	E2 binding	ERE binding	ERE activation	Activation AP1-reporter	Hetero-dimers	Ref
rER–β1	+	+	+	- Estrogens + Antiestrogens	rER–β1 rER–α rER–β2	6, 9, 10, 25
rER–β2	+ -	+	+ -		rER–β1 rER–α rER–β2	9, 10
rER–β2Δ3	+ -	-				10
rER–β1Δ3	+	-				10
mER–β1	+	+	+		hER–α mER–α	14, 16
hER–β1short	+	+	+	- Estrogens + Antiestrogens	hER–β2 hER–α hER–β3	17, 25, 30, 31, 32, 33
hER–β1long	+	+	+		hER–α	19, 20
hER–β2	-	+ -	-		hER–β1 hER–α hER–β3	20, 21
hER–β3		+			hER–β1 hER–α hER–β2	20

	rER-β1	rER-β2	rER-β1Δ3	rER-β2Δ3
Prostate	P: 43,42,44,8,10, 23, 9 I: 42,6 H: 41 R: 10	P: 8,10,9 R: 10	P: 10	P: 10
Testis	P: 44,23 H: 41,45			
Peritubular cells	H: 45			
Sertoli cells	H: 45			
Leydig cells	H: 45			
Epididymis	P: 8	P: 8		
Ovary	P: 46,8,10,23,9 N: 46 R:10	P: 8,10,9 R: 10	P: 10	P: 10
Granulosa cells	I: 46,6 H: 41			
Corpus luteum	I: 47,46 H: 41			
Oviduct	H: 41			
Uterus	P: 44,8,10,23,9 N: 48 H: 41	P: 8,10,9 R: 10		
Brain	P: 9,10 R: 10			
Pituary	P: 8,10	P: 8,10		
Supraoptic nucleus	H: 49 I: 49,50,51			
Paraventricular nucleus	H: 49 I: 49,50,51			
Hippocampus	P:10			
Hypothalamus	P: 10 I: 51	P: 10	P: 10	P: 10
Liver	P:9			
Muscle	P: 10	P: 10		
Duodenum	P: 48			
Antral Mucosa	P: 48			
Fundic Mucosa	P: 48 N: 48			
Bladder	H: 41 P: 23			
Adrenal	H: 41			
Kidney	P: 9			
Lung	H: 41 P: 23,9			
Thymus	P: 44,8 H: 41	P: 8		
Spleen	P: 44			
Bone	P: 44,9			
Heart	W: 52			
Aorta	P: 10		P: 10	P: 10

	mER-β1	mER-β2	mER-β1Δ5	mER-β1Δ5–6	mER-β1Δ6
Prostate	R: 53				
Testis	P: 40				
Elongated spermatids	H: 40				
Leydig cells	H: 40 P: 40				
Epididymis	R: 53 P: 40				
Ovary	R: 53 N: 14 P: 15	P: 15	P: 15	P: 15	P: 15
Uterus	P: 15	P: 15	P: 15	P: 15	P: 15
Mammary gland	P: 15	P: 15	P: 15	P: 15	P: 15
Hippocampus	I: 54				
Hypothalamus	R: 53 I: 54				
Paraventricular nucleus	I: 54				
Liver	R: 53				
Bladder	H: 40				
Lung	R: 53				
Heart	R: 53				

	hER-β1	hER-β2	hER-β4	hER-β5	hER-βΔ5	hER-βΔ5–6	hER-βΔ6
Prostate	P: 20	P: 20, 21		P: 20			
Testis	P: 20,55 N: 11,17	P: 20, 21	P: 20	P: 20			
Elongated spermatids	I: 11						
Ovary	P: 20,55,57,15 N: 11,17,56 I: 11	P: 20,21	P: 20	P: 20	P: 15	P: 15	P: 15
Granulosa cells	I: 11						
Uterus	P: 20,11,55,57,15	P: 20	P: 20	P: 20	P: 15	P: 15	P: 15
Breast	P: 20,11,15 I: 11		P: 20	P: 20	P: 15	P: 15	P: 15
Breast Tumor	P: 11,15,18, 58, 22 I: 11				P: 15,18	P: 22, 15	P: 15
Brain	P: 20,55						
Hippocampus	P: 59						
Hypothalamus	P: 59						
Liver	P: 20			P: 20			
Muscle	P: 20, 55	P: 20		P: 20			
Colon	P: 20,55 I: 11	P: 20		P: 20			
Duodenum	I: 11						
Small intestine		P: 20		P: 20			
Fat	P: 20	P: 20		P: 20			
Kidney	I: 11 P: 55			P: 20			
Adrenal cortex	I: 11 P: 55						
Lung	I: 11 P: 55			P: 20			
Thymus	P: 20,55 N: 17	P: 20,21	P: 20	P: 20			
Spleen	P: 20,55	P: 20	P: 20	P: 20			
Bone marrow	P: 20			P: 20			
Aorta	P: 55,57						
Heart	P: 20,55			P: 20			

APPENDIX 3

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In press Britis J cancer 15/4/98

ESTROGEN RECEPTOR-α VARIANT mRNA EXPRESSION IN PRIMARY HUMAN BREAST TUMORS AND MATCHED LYMPH NODE METASTASES.

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Running Title:

Estrogen receptor variants in breast cancer.

Key Words:

RT-PCR, RNase Protection Assay, Triple primer PCR, alternative splicing

ABSTRACT. We had shown previously that the relative expression of a truncated estrogen receptor-α variant mRNA (ER clone 4) was significantly increased in axillary node positive primary breast tumors compared to node negative tumors. In this study we have examined the relative expression of clone 4 truncated, exon 5 deleted and exon 7 deleted estrogen receptor-α variant mRNAs in 15 primary breast tumor samples and in synchronous axillary lymph node metastases. Overall there were no significant differences between the primary tumors and the matched metastases in the relative expression of these three specific variant mRNAs. Furthermore, the pattern of all deleted estrogen receptor-α variant mRNAs appeared conserved between any primary and its matched secondary tumor.

INTRODUCTION.

Multiple estrogen receptor-α (ER) mRNA species have been identified in human breast cancer samples (1, 2). The significance of these variant transcripts remains unclear. While the ability to detect variant ER proteins encoded by such variant transcripts remains controversial (3-5) alteration of expression of some variant ER mRNAs was found to occur during both breast tumorigenesis (6, 7) and breast cancer progression. With regard to the latter, we have shown previously that the expression of the truncated, clone 4 variant (C4) ER mRNA (8) was significantly increased relative to wild type (WT) ER mRNA in a group of primary breast tumors with multiple poor prognostic features compared with a group of primary breast tumors with good prognostic features (9). The "poor" prognostic features were defined as the presence of lymph node metastases at the time of surgery, large tumor size, lack of progesterone receptor (PR) expression and high S-phase fraction, while "good" prognostic features were lack of nodal involvement, small tumor size, PR positivity and low S-phase fraction. In the same study, the relative expression of clone 4 ER

variant mRNA was significantly higher in primary breast tumors which were PR- compared to those which were PR+ (9). This suggested that altered ER variant expression may be a marker of a more aggressive phenotype and lack of endocrine sensitivity in human breast cancer. As a prerequisite to addressing such a possibility we have investigated the pattern of ER variant expression in a cohort of primary tumors and their matched, concurrent lymph node metastases.

MATERIALS AND METHODS.

Tumor Selection and RNA Isolation.

Sections from 15 frozen primary human breast tumor samples and their matched frozen lymph node metastases were provided by the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). For the primary tumor samples, the ER levels, determined by ligand binding assays, ranged from 0.8 fmol/mg protein to 89 fmol/mg protein with a median value of 17.5 fmol/mg protein. Thirteen tumors were ER+ and 2 were ER- (ER+ was defined as > 3 fmol/mg protein). PR levels determined by ligand binding assays ranged from 2.9 fmol/mg protein to 112 fmol/mg protein with a median value of 12.6 fmol/mg protein. Nine tumors were PR+ and 6 were PR- (PR+ was defined as > 10 fmol/mg protein). ER and PR values were only available for 4 of the lymph node metastases and the ER and PR status as defined by ligand binding did not differ from their matched primary tumor. RNA was extracted from the sections using Trizol reagent (Gibco/BRL, Ontario, Canada) according to the manufacturer's instructions.

For validation of triple primer polymerase chain reactions (TP-PCR) by comparison with RNase protection assays, a second cohort of human breast tumor specimens (25 cases) was also obtained from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Twenty of these tumors were ER+, as determined by ligand binding assay, with values ranging from 4.5 to 311

fmol/mg protein (median 93 fmol/mg). The five remaining cases were ER- with values ranging from 0 to 1.8 fmol/mg protein (median 0.9 fmol/mg). Total RNA was extracted from frozen tissues using guanidinium-thiocyanate as previously described (10). The integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described (10).

RNase Protection Assay.

Antisense riboprobes spanning the point at which the C4 ER mRNA sequence diverges from the WT ER mRNA sequence (8) were synthesized as previously described (11). The level of C4 ER mRNA and WT ER mRNA in 10 µg total RNA was determined using an RNase Protection Assay kit (RPA II, Ambion, Austin, Texas) following the manufacturer's instructions. Briefly, RNA was denatured at 80°C for 5 min in the presence of 5 x 10^5 dpm of ^{32}P-labelled riboprobe, then hybridized at 42°C for 16 hrs. Following RNase digestion, samples were electrophoresed on 6% acrylamide gels containing 7 M urea, dried and autoradiographed.

To quantify C4 and WT ER mRNAs within breast tumor samples, a standard curve was established in each assay. C4 and WT ER mRNAs (30, 125, 500 pg C4 RNA and 125, 500, 2000 pg WT ER RNA) synthesized using T7 RNA polymerase were purified on a Sephadex G-50 column and quantitated spectrophotometrically. WT ER RNA was transcribed from linearized pHEO, which contains the entire WT ER coding sequence but is missing the 3'-untranslated portion of the ER mRNA (kindly provided by P. Chambon, Strasbourg, France (12)). Full-length C4 RNA was transcribed from linearized pSK-C4 (8). Standard RNAs were analyzed together in the same assay as the breast tumor mRNAs. Bands corresponding to the C4 ER mRNA and WT ER mRNA protected fragments were excised from the gel and counted after addition of 5 ml scintillant (ICN Pharmaceuticals, Inc., Irvine, CA) in a

scintillation counter (Beckman Instruments, Inc., Fullerton, CA). For each sample, absolute amounts of C4 and WT ER mRNA were determined from the standard curve.

Reverse transcription, PCR and Triple Primer (TP)-PCR.

For each sample, 1 µg total RNA was reverse transcribed in a final volume of 15 µl as described previously (7). One microliter of the reaction mixture was taken for subsequent amplification.

The primers and PCR conditions for the long range PCR were as previously described (13). The primers and PCR conditions for measuring the relative expression of exon 5 deleted and exon 7 deleted ER transcripts relative to WT ER transcripts were as previously described (7).

The TP-PCR conditions were similar to those previously described (6) with minor modifications. ERU (5'-TGTGCAATGACTATGCTTCA-3', sense, located in WT ER exon 2; 792-811, as numbered in (12)) and ERL (5'-GCTCTTCCTCCTGTTTTTAT-3', antisense, located in WT ER exon 3; 921-940) primers allowed amplification of a 148 bp fragment corresponding to WT ER mRNA. The C4 specific primer (C4L, 5'-TTTCAGTCTTCAGATACCCCAG-3', antisense; 1315-1336, as numbered in reference (8)) spans the only region of the C4 unique sequence that does not have any homology with repetitive LINE-1 sequences (8). ERU and C4L allowed amplification of a 536 bp fragment corresponding specifically to C4 ER variant mRNA.

PCR amplifications were performed in a final volume of 10 µl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/µl of each primer (ERU, ERL and C4L), 0.2 units of Taq DNA polymerase (GIBCO-BRL) and 1 µCi of dCTP [α-^{32}P] (3000 Ci/mmol, ICN Pharmaceuticals Inc, Irvine, California). Each PCR consisted of 30 cycles (1 min at 94°C, 30 sec at 60°C and 1 min at 72°C) using

a Thermocycler (Perkin Elmer). Four µl of the reaction were then denaturated by addition of 6 µl of 80% formamide buffer and boiling before electrophoresis on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with two intensifying screens for 2 hours.

Quantification of RT-PCR and TP-PCR.

Bands corresponding to the variant ER mRNA and WT ER mRNA were excised from the gel and counted after addition of 5 ml scintillant in a scintillation counter. The variant signal was expressed as a percentage of the WT ER signal. It should be noted that the percentage obtained reflects the relative ratio of the variant to WT ER RT-PCR product and does not provide absolute initial mRNA levels. Validation of this approach was described previously (6, 7, 14, 15). At least two independent PCR assays were performed for each sample in the comparison of RNase Protection Assay with TP-PCR assays. For assessment of matched primary and secondary breast tumor samples, at least two and in most cases three independent PCR reactions were performed and the mean determined.

The statistical significance of differences in the relative levels of expression of any single ER mRNA variant between primary tumor and lymph node metastasis was determined using the Wilcoxon signed rank test.

RESULTS.

Multiple ER variant mRNAs have been shown to be expressed in any one breast tissue sample (1, 7, 16). To investigate the pattern of multiple exon deleted ER variant expression between primary breast tumors and their matched lymph node metastases a long range RT-PCR approach was used. This approach, based on the competitive amplification of wild type and exon deleted ER variant cDNAs using primers annealing within exons 1 and 8, allows

the evaluation of the relative pattern of expression of all exon deleted ER variant transcripts present in any individual sample (13, 17). Typical results are shown in Figure 1A. The pattern of deleted ER mRNAs expression between any one primary tumor and its matched lymph node metastasis was conserved. Using a previously validated semi-quantitative PCR approach (7) the measurement of the relative expression of specific individual exon deleted ER variant mRNAs was also undertaken. Specifically, the relative expression of exon 5 deleted ER cDNA (Figure 1B) using primers in exons 4 and 6, and exon 7 deleted ER cDNA (Figure 1C) using primers in exons 5 and 8, were measured. The median value for the relative expression of the exon 5 deleted ER for the primary tumors was 23.1 % (range 17.3% - 94.3%) and the median value for the matched lymph node metastases was 31.3 % (range 14.9 % - 200%). The scatter plot for these results is shown in Figure 2A. The median relative expression of the exon 7 deleted ER for primary tumors was 65% (range 39.3% - 184.9%) and the median value for the matched lymph node metastases was 52.5% (range 35.5% - 126%). The scatter plot of these results is shown in Figure 2B. There were no statistically significant differences in the relative expression of either exon deleted ER mRNA between primary and concurrent metastatic tumors.

Another frequently expressed ER variant, which would not be detected in the above assays, is the C4 ER mRNA. This variant was previously found to be significantly elevated in a group of primary breast tumors with poor prognostic features which included concurrent lymph node metastases, compared with a group of primary tumors with good prognostic variables which included lack of concurrent nodal metastases (9). Therefore, it was relevant to determine the level of C4 ER variant expression in primary breast tumors and their matched, concurrent lymph node metastases.

In this previous study we used RNase protection assays to measure WT and variant ER mRNA expression (9). However, in order to conduct this study using smaller tissue samples (in particular from nodal metastases) and to ensure a close correlation with the histological composition of the tissue, we used a previously described TP-PCR assay (6) to measure the relative expression of C4 ER mRNA. To facilitate comparison of the current data with our earlier study (9), it was necessary to compare the RNase protection assay with the TP-PCR assay, before proceeding to analyze the primary and secondary breast tumor samples for C4 mRNA expression by TP-PCR.

RNA from 25 human breast tumors, selected to represent a wide range of ER status by ligand binding assay (Table 1), was analyzed in a standardized RNase protection assay in order to determine the absolute amount of C4 and WT ER mRNAs within each sample. The signals corresponding to C4 and WT ER mRNAs were quantified as described in Materials and Methods. In each assay, known amounts of synthetic WT ER and C4 mRNAs were analyzed in parallel in order to establish a standard curve allowing the determination of absolute levels of C4 and WT ER mRNAs, expressed as pg/10 µg RNA (Table 1). Because of the very low C4 protected fragment signal (≤ 15 dpm) in seven tumors, it was not possible to determine confidently the absolute amount of C4 mRNA in these samples (not determined, nd). All C4 negative tumors by RNase protection assay were from tumors with ER values lower than 10 fmol/mg protein, as determined by ligand binding assay. The absolute amounts of C4 and WT ER mRNAs in the remaining 18 tumors as determined by RNase protection assay, varied from 2 to 83.9 pg/10 µg RNA and from 9 to 3651 pg/10 µg RNA, respectively. For each sample, the C4 mRNA signal was expressed as a percentage of WT ER mRNA signal (Table 1).

C4 ER mRNA relative expression was determined by TP-PCR within the same 25 RNA samples as described in Materials and Methods. Both C4 and WT ER cDNAs signals were detected in all 25 tumors studied, independent of their ER status as determined by ligand binding assay. C4 and WT ER signals were quantified as described in Materials and Methods. The signal corresponding to C4 was expressed as a percentage of the WT ER signal. Table 1 presents the average of a least two independent TP-PCR experiments. Linear regression analysis (Figure 3) shows a highly significant correlation between C4 mRNA relative expression as determined by RNase protection assay (in the 18 tumors where a C4 signal was detectable) and C4 mRNA relative expression determined by TP-PCR ($r = 0.932$, $P < 0.0001$). Interestingly, an additional band was also observed in most of the samples using the TP-PCR assay (see * Figure 1D). This band was identified after subcloning and sequencing to be a product of an exon 2 duplicated ER variant mRNA. The intensity of the signal obtained from this exon 2 duplicated ER band paralleled that of the WT ER band and the co-amplification of the exon 2 duplicated ER variant mRNA using TP-PCR did not interfere with the relationship between TP-PCR and RNase protection assay.

The above TP-PCR assay was used to compare the relative expression of C4 and WT ER expression in the matched breast cancer samples (Figure 1D). The median relative expression of the C4 ER for the primary tumors was 3.5 % (range 1.6 - 10.5%) and the median value for the matched lymph node metastases was 3.1 % (range 1.0 - 19.4%). A scatter plot of the results is shown in Figure 2C. There is no statistically significant difference in the relative expression of C4 ER variant expression between primary breast tumors and their concurrent lymph node metastases by Wilcoxon rank sum analysis. Interestingly, although not statistically significant, we found that the median level

of C4 expression in ER+ PR- primary tumors, 3.7% (range 2.5 - 7.9%, n = 5), was approximately 50% higher than the median level of C4 expression in ER+ PR+ primary tumors, which was 2.4% (range 1.6 -10.5%, n = 8). Such a trend would be consistent with our previous results in which C4 expression was higher in PR- primary breast tumors compared with PR+ primary tumors.

DISCUSSION.

The data presented in this study provide evidence that both the overall pattern of ER variant expression and the relative level of expression of three individual ER variants are conserved in primary breast tumors and their matched, concurrent lymph node metastases.

The observations presented in this manuscript showing a conserved pattern and similar relative expression of ER variants between primary tumors and their concurrent lymph node metastases would be consistent with previous observations that little change of ER status can be found between primary human breast tumors and their concurrent lymph node metastases or their distant metastases (18, 19). These findings are also not inconsistent with our previously published data in which the relative expression of at least one ER variant was significantly increased in primary tumors with poor prognostic characteristics, which included having concurrent lymph node metastases, as compared to primary tumors without concurrent lymph node metastases (9). The primary tumors in the current study have concurrent lymph node metastases, a major feature of poor prognosis in breast cancer, and most likely resemble the previously described poor prognostic group. Our earlier observation of higher relative C4 ER mRNA expression in PR- primary tumors versus PR+ primary tumors appeared to be conserved in this cohort although the numbers were low and the difference did not reach statistical significance. Since altered expression of several ER variants has been shown to occur in primary breast

tumors compared to normal human breast tissues (6, 7), as well as between good versus poor prognosis primary breast tumors, the current data suggest that alterations of ER variant expression and any role this may have in altered estrogen signal transduction likely occurs early in tumorigenesis and well before the acquisition of the ability to metastasize. This is consistent with previous data supporting the concept of an early involvement of perturbations of estrogen signal transduction and the development of hormone independence in breast tumorigenesis (20, 21). It remains therefore to be determined if altered ER variant expression can predict tumor recurrence and progression in node negative breast cancers.

To our knowledge this study is the first that addresses the question of the comparison of an already established quantitative approach such as the RNase protection assay with an RT-PCR based approach in the study of ER variant mRNA expression. Earlier studies have either been done by RNase protection assay alone or by RT-PCR alone. Considering the potential clinical relevance of the measurement of the relative level of ER variants with respect to WT ER within human breast tissue samples and the sensitivity of an RT-PCR based approach, such a comparative study was deemed necessary. Furthermore, our data provide validation for comparing previous data obtained using a non-amplification dependent RNase protection assay with the current data obtained using an amplification dependent TP-PCR assay.

The lack of sensitivity of the RNase protection assay for a subset of tumors with very low (<10 fmol/mg) ER values by ligand-binding assay is an important limiting factor. It effectively means that in a screening study, ER negative tumors (<3 fmol/mg protein) as well as ER positive tumors with ER values lower than 10 fmol/mg, as measured by ligand binding assay, cannot be reliably assessed for C4 ER variant mRNA expression by RNase protection

assay. This together with the relatively large amount of RNA needed to perform an RNase protection analysis severely limits the usefulness of a standardized RNase protection assay in such screening studies. The low amount of starting material needed, together with the higher sensitivity observed (samples C4 ER variant negative by RNase protection assay had detectable levels of C4 ER variant and WT ER mRNA by TP-PCR) make TP-PCR an attractive alternative to the RNase protection assay in studies where such factors are limiting.

In conclusion, the current investigation extends our previous studies on the relationship of ER variant expression and progression in human breast cancer. The data presented show that both the pattern and level of expression of ER variants is conserved between matched primary breast tumors and their concurrent lymph node metastases. Therefore, any alteration of ER variant expression which could be a marker of altered ER signal transduction and breast cancer progression, likely occurs before breast cancer cells acquired the ability to metastasize.

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LEGENDS TO FIGURES.

Figure 1:

A. Autoradiogram of long range RT-PCR (13) results from two samples of primary breast tumors (P) and their matched concurrent lymph node metastasis (M). WTER is the expected product corresponding to the wild type ER mRNA; D7 is the expected product corresponding to the exon 7 deleted ER variant mRNA; D4 is the expected product for the exon 4 deleted ER mRNA; D3-4 is the expected product for the exon 3+4 deleted ER mRNA; D4/7 is the expected product for the exon 4 and exon 7 deleted ER mRNA.

B. Autoradiogram of RT-PCR results from two samples of primary breast tumors (P) and their matched concurrent lymph node metastasis (M). D5 is the expected product corresponding to the exon 5 deleted ER variant mRNA. WTER is the expected product corresponding to the wild type ER mRNA.

C. Autoradiogram of RT-PCR results from two samples of primary breast tumors (P) and their matched concurrent lymph node metastasis (M). D7 is the expected product corresponding to the exon 7 deleted ER variant mRNA. WTER is the expected product corresponding to the wild type ER mRNA.

D: Autoradiogram of TP-PCR results from two samples of primary breast tumors (P) and their matched concurrent lymph node metastasis (M). C4 is the expected product corresponding to the clone 4 ER variant mRNA. WTER is the expected product corresponding to the wild type ER mRNA. * indicates a band coamplified with C4 and wild type ER and shown to correspond to an exon 2 duplicated ER variant mRNA.

Figure 2:

A. Quantitative comparison of the relative expression of exon 5 deleted variant ER mRNA in primary (P) human breast tumors and their concurrent matched lymph node metastases (M). For each sample the mean of three

independent measures of exon 5 deleted ER relative expression expressed as a percentage of the corresponding wild type ER signal was determined as described in the Materials and Methods section.

B. Quantitative comparison of the relative expression of exon 7 deleted variant ER mRNA in primary (P) human breast tumors and their concurrent matched lymph node metastases (M). For each sample the mean of three independent measures of exon 7 deleted ER relative expression expressed as a percentage of the corresponding wild type ER signal was determined as described in the Materials and Methods section.

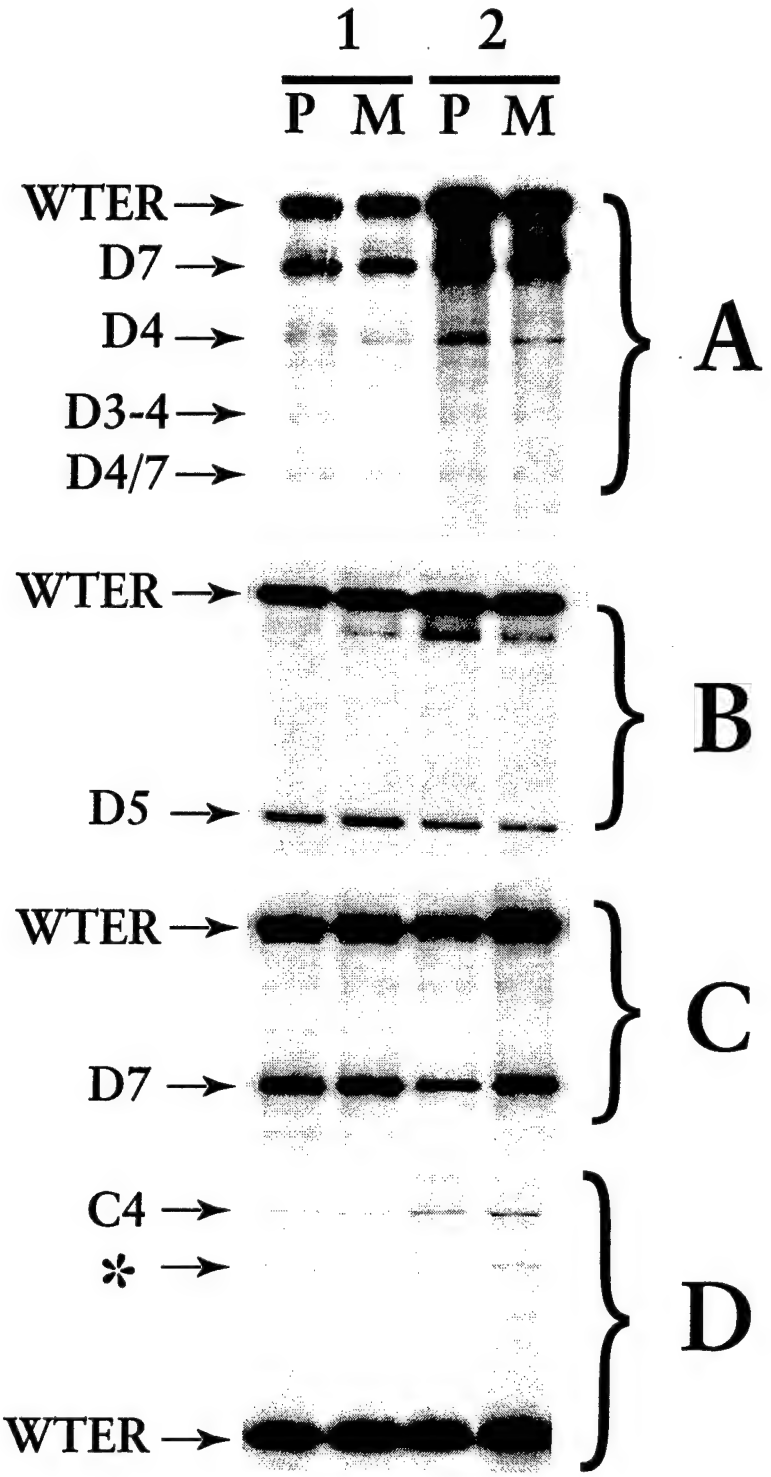
C. Quantitative comparison of the relative expression of clone 4 variant ER mRNA in primary (P) human breast tumors and their concurrent matched lymph node metastases (M). For each sample the mean of three independent measures of clone 4 relative expression expressed as a percentage of the corresponding wild type ER signal was determined as described in the Materials and Methods section.

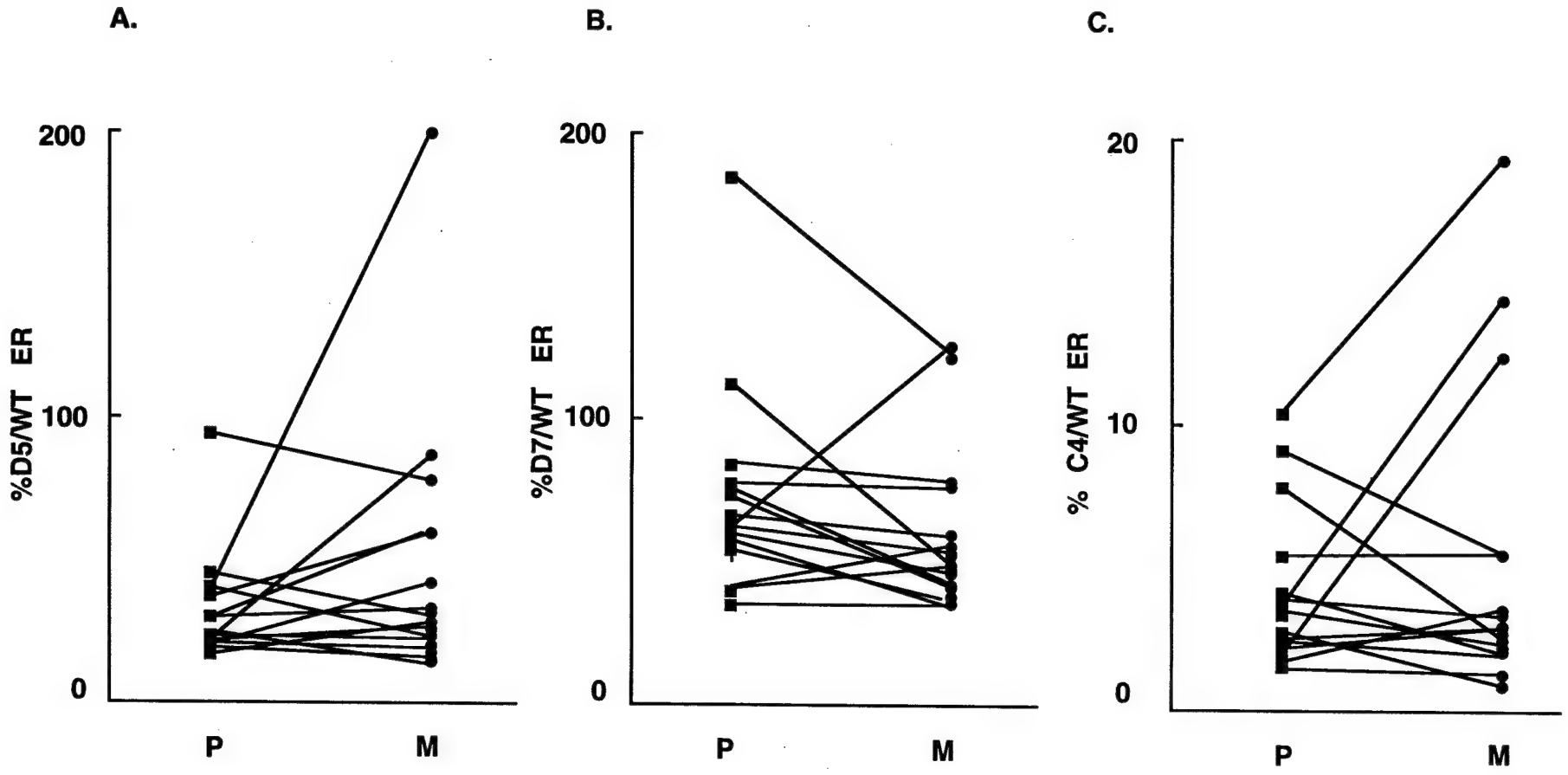
Figure 3:

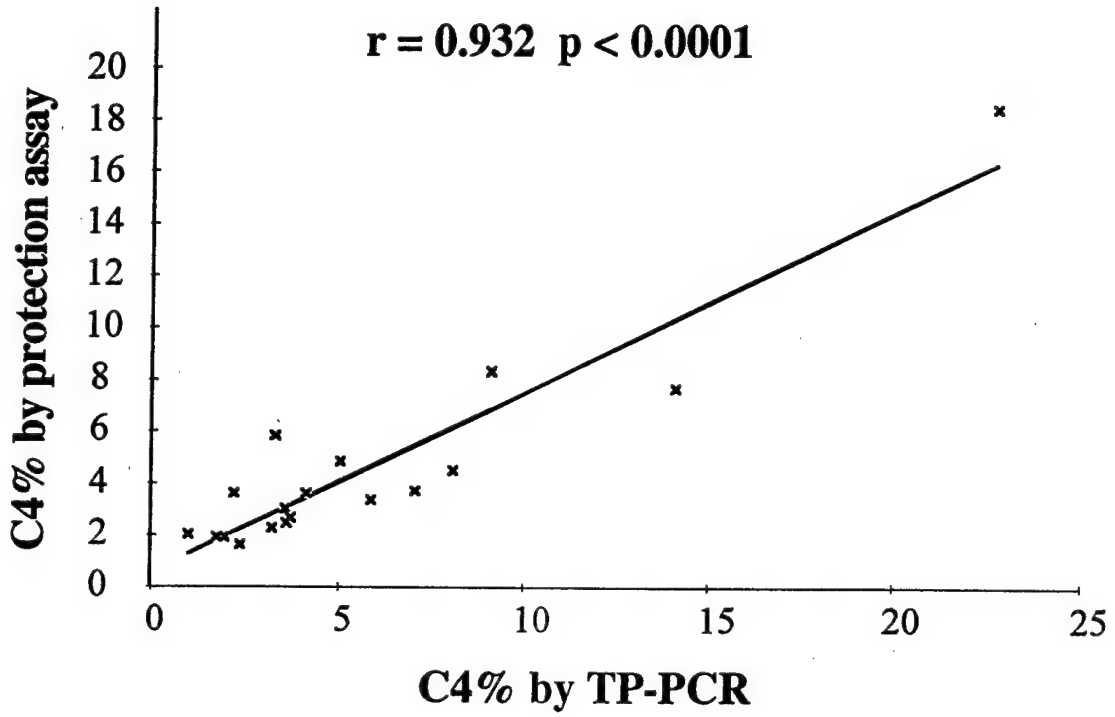
Linear regression analysis of clone 4 expression (expressed as a percentage of the corresponding wild type ER expression) as determined by TP-PCR versus standardized RNase protection assay in eighteen human breast tumors.

Table legends

Table 1: C4 and WT-ER mRNA expression in twenty five human breast tumors, as determined by RNase protection assay and TP-PCR


1
2
P M P M
WTER
D7
D4
D3-4
D4/7
A
WTER
D5
B
WTER
D7
C
C4
*
WTER
D


A.
B.
C.
200
100
0
%D5/WT ER
%D7/WT ER
20
10
% C4/WT ER
P
M


r = 0.932 p < 0.0001
C4% by protection assay
C4% by TP-PCR
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
25

Sample N°	Ligand Binding	RNase protection			TPPCR
	ER fmole mg	C4 pg/10µg	WT-ER pg/10µg	C4 %	C4 %
5	0.0	nd[a]	nd	-	1.7
3	0.4	nd	nd	-	2.6
1	0.9	nd	nd	-	3.1
24	1.2	6.2	105.1	5.9	3.3
4	1.8	nd	nd	-	3.7
23	4.5	10.0	54.3	18.4	22.7
8	5.8	nd	26.8	-	2.8
7	6.3	nd	224.6	-	3.4
2	8.7	nd	9.0	-	2.2
19	10.0	22.6	902.9	2.5	3.6
10	17.8	5.3	146.4	3.6	4.1
13	25.0	2.3	112.0	2.0	1.0
15	44.0	5.0	148.5	3.4	5.9
22	57.0	11.8	153.6	7.7	14.1
11	90.0	2.5	129.1	1.9	1.7
21	96.0	9.6	263.4	3.6	2.2
14	105.0	4.6	94.4	4.9	5.0
17	111.0	26.7	320.3	8.3	9.1
9	121.0	4.6	277.7	1.7	2.4
6	146.0	2.0	105.0	1.9	1.9
18	198.0	15.8	422.0	3.7	7.0
20	236.0	8.8	288.4	3.0	3.5
12	289.0	3.6	80.5	4.5	8.0
16	304.0	38.8	1440.8	2.7	3.7
25	311.0	83.9	3651.0	2.3	3.2

APPENDIX 4

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Variant estrogen receptor-alpha messenger RNA expression in hormone-independent human breast cancer cells.

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Running title: Variant estrogen-receptor alpha and estrogen-independence.

Key words: Estrogen receptor, estrogen-independence, variant, breast cancer, exon deletion.

Abstract

The development of estrogen-independent growth is thought to be an important step in the progression of breast cancer to hormone-independence and endocrine therapy resistance. T5 human breast cancer cells are estrogen receptor (ER-α) positive and estrogen treatment in culture results in increased proliferation of these cells. An estrogen-nonresponsive cell line, T5-PRF, was developed from T5 cells, by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen while still retaining expression of the ER-α. In the apparent absence of ligand T5-PRF cells have a 3.6 ± 0.5 fold increased basal ER-α transcriptional activity and elevated basal progesterone receptor (PR) levels compared to the parent T5 cells. Long range ER-α reverse transcription-polymerase chain reaction (RT-PCR) was performed to characterize the pattern of variant ER–α mRNA expression between the two cell lines and a differential expression of an ER-α mRNA variant was found. In particular, an ER–α variant mRNA, deleted in exons 3 and 4, was detected only in T5-PRF cells. Recombinant expression of this ER–α variant confered increased basal transcriptional activity and estrogen-responsiveness when expressed with wild-type ER-α in ER negative cell lines, as well as increasing both ligand-independent and estrogen-induced ER-α transcriptional activity when expressed alone in parental T5 cells. These results suggest a possible role for the altered expression of an ER-α variant in ligand-independent activation of ER-α which may contribute to the estrogen-independent phenotype in T5-PRF human breast cancer cells.

Introduction

Breast cancer is a hormonally responsive cancer and hormones, including estrogen, are required for breast cancer growth (1). Estrogens promote the growth of human breast cancer, and as such, most endocrine therapies are aimed at blocking the growth promoting effects of estrogen (e.g., antiestrogen such as tamoxifen). Breast cancers are classified according to their requirement for proliferation as being either hormone-dependent or hormone-independent, based ultimately on the response to endocrine therapy of metastatic disease (2). The level of estrogen receptor-alpha (ER-α) in human breast cancer (HBC) is used as a marker not only of potential therapeutic response to endocrine therapy, but is a marker of prognosis and survival (3).

The evolution of breast cancer into an estrogen-independent growth phenotype is thought to be an important step in the progression of breast cancer to hormone-independence and endocrine therapy resistance (4, 5). Understanding the factors that contribute to the development of a hormone-independent phenotype is of major importance in terms of breast cancer therapeutics. Resistance to endocrine therapies may be due to a number of factors. In some cases, hormone-independence and resistance can occur due to loss of ER expression, but most tumours that have developed resistance to endocrine therapy remain receptor positive (6).

Several breast cancer cell lines in culture also require estrogen for growth and long-term culture in estrogen-depleted conditions can result in cells becoming apparently independent of the requirement for estrogen for growth. Indeed, the development of estrogen-independent growth in human breast cancer is thought to be one of the initial steps in the progression to hormone-independence and resistance to endocrine therapies (7). However, the mechanisms responsible for the development of estrogen-independence in the presence of continued expression of ER-α are poorly understood. In order to address this we have developed a breast cancer cell model of apparent estrogen independence (8). T5 human breast cancer cells are ER-α positive and estrogen treatment in culture results in

increased proliferation of these cells. An estrogen-nonresponsive cell line, T5-PRF, was developed from T5 cells by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen seen in the parent cell line while still retaining expression of the ER-α (8). However, these cells remain sensitive to the growth inhibitory effects of 4-hydroxy-tamoxifen (OT) and ICI 164,384 (ICI), although they have reduced sensitivity to ICI compared to the parent T5 cells (8).

In this study we have investigated the ligand-dependent and -independent transcriptional activity of the endogenous ER-α as well as the pattern and potential function of ER-α variant expression in T5 and T5-PRF human breast cancer cells.

Materials and Methods

Materials

[^{32}P]dCTP and [^{35}S]ATP were purchased from ICN (St-Laurent, Quebec). Dulbecco's Minimal Essential Medium (DMEM) powder and fetal bovine serum were purchased from GIBCO/BRL (Burlington, Ontario). Horse serum and EGF were purchased from UBI (Lake Placid, New York). All other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario). Cholera toxin, 4-hydroxy-tamoxifen, estradiol-17β and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). [^{14}C]-chloramphenicol, [^{3}H]- R5020 (88.7 Ci/mmol), [^{35}S]-methionine and R5020 were obtained from NEN (Lachire, Quebec). ICI 164,384 was a gift from ICI (Macclesfield, Cheshire).

Cells and Cell Culture

T5 cells, previously called T-47D5, were originally thought to be a T-47D subline, however, DNA fingerprinting analysis showed that they were an MCF-7 subline (9). T5 and MDA-MB-231 human breast cancer cells were routinely cultured in DMEM containing 5% vol/vol fetal calf serum, 1% wt/vol glucose, glutamine and penicillin-streptomycin. T5-PRF cells were routinely cultured in phenol red-free DMEM supplemented with 5% vol/vol twice charcoal dextran stripped fetal calf serum and 1% wt/vol glucose, glutamine, and penicillin-streptomycin (PRF/DMEM). MCF10A1 human breast epithelial cells (10) were grown routinely in DMEM containing 5% vol/vol horse serum, 1% wt/vol glucose, glutamine and penicillin-streptomycin, 0.1μg/ml cholera toxin, 20ng/ml hEGF, 10.4μg/ml bovine insulin and 1μM hydrocortisone (DMEM-special). Transient transfections and steroid receptor assays were performed in PRF/DMEM. Transient transfections using MCF10A1 cells were performed in phenol red-free DMEM containing 5% vol/vol charcoal-stripped horse serum, 1% wt/vol glucose, glutamine and penicillin-streptomycin (PRF/DMEM-hs) and cells were passaged once prior to transfection in phenol red-free DMEM containing 5% vol/vol charcoal-stripped horse serum, 1% wt/vol glucose, glutamine, penicillin-streptomycin, 0.1μg cholera toxin, 20ng/ml hEGF, 10.4μg/ml bovine insulin and 1μM hydrocortisone (PRF/DMEM-special).

Progesterone Receptor Assays

PR assays were performed using whole cell ligand binding assays as previously described (11). [^{3}H]-R5020 and [^{3}H]-R5020 and 100 fold molar excess unlabelled R5020 were used to determine PR total and nonspecific binding, respectively. All assays were performed in the presence of 100 nM dexamethasone to prevent binding of R5020 to the glucocorticoid receptor.

Transient transfections and CAT assays

T5, T5-PRF and MDA-MB-231 cells were passaged once in PRF/DMEM and set up in 100 mm diameter dishes at 0.5 X 10^6 cells per dish in PRF/DMEM the day before transfection. MCF10A1 cells were passaged once in PRF/DMEM-special and set up in 100 mm diameter dishes at 2 X 10^6 cells per dish in PRF/DMEM-special two days before transfection. The following day the medium was changed to PRF/DMEM-hs and cells were transfected the following day, using the calcium phosphate/glycerol shock method (12) using an equal volume 2 x BBS buffer (50mM BES, 280mM NaCl, 1.5mM Na_2HPO_4 pH 6.95), followed by a 2 minute glycerol shock (20% vol/vol). Cells were washed twice with PBS and given fresh medium plus or minus 10 nM estradiol-17β (E2), 10nM estradiol-17β plus 1μM ICI 164,384 or 1μM ICI 164,384 alone. After 24h of treatment, the cells were harvested, cell extracts prepared and chloramphenicol acetyltransferase (CAT) activity measured (13). Transfection efficiency was determined by cotransfection of pCH110 (β-galactosidase expression vector, Pharmacia) and assay of β-galactosidase activity (14). T5 and T5-PRF cells were transfected with 5μg of ERE-tk-CAT (15), to determine ER–α transcriptional activity, along with 5μg pCH110. In the experiments where activity of d3/4 was examined in T5 cells, transfections were performed using 5μg ERE-tk-CAT, 5μg pCH110 plus or minus d3/4 expression vector (0.1-1 pmol) or vector DNA alone. MDA-MB-231 and MCF10A1 cells were transfected with 5μg ERE-tkCAT, 5μg pCH110, plus or minus 0.5pmol HEGO (wild-type ER-α expression vector, kindly provided by Dr P.

Chambon) with increasing amounts (0.5-2 pmol) of d3/4 expression vector or vector DNA alone.

Long-Range ER-α RT-PCR

Total RNA was extracted (Trizol reagent, GibcoBRL, Grand Island, NY) and reverse transcribed as described previously (16). The primer pair used consisted of 1/8U primer (5'-TGCCCTACTACCTGGAGAACG-3', sense; located in WT-ER-α exon 1; nucleotides 615-637) and 1/8L primer (5'-GCCTCCCCCGTGATGTAA-3', antisense; located in WT-ER-α exon 8; nucleotides 1995-1978). Nucleotide positions given correspond to published sequences of the human ER-α cDNA (17). PCR amplifications were performed as previously described (18). PCR products were separated on 3.5% polyacrylamide gels containing 7M urea, gels were dried and labelled products visualized by autoradiography. PCR products were subcloned and sequenced as previously described (16).

Construction of variant ER-α expression vector

The RT-PCR product corresponding to the exon 3/4 deleted ER-α cDNA was cloned into the TA cloning vector (Invitrogen TA cloning kit). Stu I digestion of this plasmid released an exon 3/4 deleted fragment which was used to replace the corresponding region of wild-type ER-α from p0R8 (19) (contains a glycine to valine point mutation at amino acid 400). Stu I sites are in exon 2 and 7 of wild-type ER-α and the subcloned d3/4 PCR fragment resulted in a correction to the wild-type sequence of glycine at amino acid 400 (in exon 5 in pOR8). The full length EcoR I ER-α fragment from HEGO (an expression plasmid containing wild-type ER-α coding region cloned into the eukaryotic expression vector pSG5, a gift from Dr. P. Chambon, Strasbourg, France (19)) was then excised and replaced with the corresponding fragment from pOR8 containing the exon 3/4 deleted ER-α cDNA. The identity of the expression plasmid containing the exon 3/4 deleted ER-α (called d3/4) was confirmed by restriction enzyme digest and sequence analysis.

In vitro transcription and translation

In vitro transcription/translation reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System, Promega, Madison, WI). Reactions were performed according to the manufacturer's instructions.

Western blotting and immune detection

Whole cell extracts (dissolved in 8M urea) were analysed using 10% SDS-PAGE with a 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method (20). Gels were transferred to nitrocellulose using CAPS transfer buffer (10 mM CAPS, pH 11, 20% methanol) and transferred for 1 hr at 120 V at 4°C. Blots were blocked for 1 hr at room temperature in 5% skimmed milk/Tris-buffered saline containing 0.5% Tween-20. Blots were incubated with either: ER-α specific primary antibody, H226, (a generous gift from Dr G. Greene, University of Chicago, IL which recognizes an epitope in exon 1/exon 2 region of the wild-type ER-α) or the ER-α specific antibody, AER 308, (Neomarkers, CA, which recognizes an epitope in exon 4 of the wild-type ER-α), overnight at 4°C in 1% skimmed milk/Tris-buffered saline containing 0.5% Tween-20. Blots were then incubated with the appropriate horse radish peroxidase conjugated secondary antibody for 1 hr at room temperature in 1% skim milk/Tris-buffered saline containing 0.5% Tween-20. Detection was carried out using the ECL detection system according to the manufacturer's instructions (Amersham, Buckinghamshire, England).

Statistical Analysis

Statistical analyses of ER-α transcriptional activity and PR levels were performed using paired Student's t-test. Statistical analysis on the effects of d3/4 on ERE-tk-CAT activity in T5 cells was performed using Wilcoxon's rank sum test. Statistical analysis on the effects of d3/4 on ERE-tk-CAT activity in MDA-MB-231 and MCF10A1 cells was performed on natural log transformed data using paired Student's t-test. Statistical analyses were performed with the help of M. Cheang, University of Manitoba, Biostatistical Consulting Unit.

Results

Apparent ligand-independent (basal) activity of ER-α is increased in T5-PRF cells.

Previously, we have shown that T5-PRF cells are no longer growth responsive to estrogen in culture, while still retaining expression of the ER-α (8). To investigate further the mechanism(s) responsible for estrogen-nonresponsiveness in these cells, ER-α transcriptional activity was examined by transient transfection assays using an estrogen-responsive reporter gene. The histogram in figure 1A represents the fold difference in chloramphenicol acetyl transferase (CAT) activity between T5 and T5-PRF cells. As expected, estrogen treatment increases CAT activity in T5 and to a lesser extent in T5-PRF cells, while the antiestrogen ICI 164,384 inhibits the estrogen induced transcriptional activity of the ER-α in both cell lines. In the absence of added estrogen there is a low basal ER-α activity in parental T5 cells, however in the estrogen-nonresponsive T5-PRF cells, the basal ER-α activity was 3.6 ± 0.5 (mean ± sem, n=7) fold higher than that seen in T5 cells ($p<0.05$). Consistent with the increased basal CAT activity in T5-PRF cells being mediated by ER-α, treating cells under basal conditions with ICI alone, completely abolished the increased basal transcriptional activity (Figure 1B).

Progesterone receptor levels are elevated in T5-PRF cells.

PR expression is a marker of ER-α activity (21), therefore we examined PR levels to determine if the increase in basal ER-α activity in T5-PRF cells was reflected in an endogenous estrogen-responsive gene. Under basal (i.e. no added estrogen) conditions the T5-PRF cells have significantly higher PR levels (~3 fold) than the parent T5 cells passaged twice in PRF/DMEM (as defined in Methods) before receptor assays (464 ± 20 fmol/10^6 cells vs 148 ± 40 fmol/10^6 cells, mean ± sem, n=3, see figure 2). Previously, we had shown that T5-PRF cells retained expression of ER-α which was approximately 50% of the levels seen in the parent T5 cells (8). Since we observed increased basal activity from both an estrogen-responsive reporter gene and an endogenous estrogen-responsive gene (PR) in T5-PRF cells despite a decreased level of endogenous ligand-binding ER, we reasoned that the

intrinsic activity of the wild-type ER in these cells was increased or some ER-like activity existed that was not detectable by ligand binding experiments.

Expression of a variant ER-α mRNA deleted in exons 3 and 4 is increased in T5-PRF cells.

Alterations in the structure or presence of variant forms of the ER-α with ligand-independent activity could be one mechanism for our observed results. Long-range ER-α RT-PCR analysis (18) was performed on RNA isolated from T5 and T5-PRF cells to examine the pattern of deleted variant ER-α mRNA expression. RT-PCR analysis was performed, using a primer pair specific for exons 1 and 8 of wild-type human ER-α sequence, allowing detection of any variant ER-α mRNA species containing both exons 1 and 8 of wild-type ER-α sequence. Figure 3A shows the PCR products obtained and the presence of a 928 bp band that is markedly increased in T5-PRF estrogen-independent cells. To confirm the identity of this variant, the cDNA corresponding to the 928 bp band was subcloned and sequenced. The nucleotide sequence of the cDNA was found to represent a variant ER-α mRNA containing a deletion of both exons 3 and 4 (Figure 3B). The exon 3 and 4 deleted ER-α (d3/4) is in frame and is predicted to encode a protein of 443 amino acid residues with a predicted molecular mass of ~49 kDa. This putative ER-α-like protein would be missing the second zinc finger of the ER-α DNA binding domain, the hinge region and part of the ligand binding domain.

The exon 3/4 deleted ER-α-like protein increases basal and estrogen-regulated wild-type ER-α transcriptional activity.

To address the potential function of this variant ER-α mRNA eukaryotic expression vectors containing d3/4 cDNA were constructed and shown to express a protein of the appropriate size (Figure 4A), that was recognized by the ER-α antibody H226 that recognizes an epitope encoded in exon 1/2 (A/B region) of wild-type ER-α (Figure 4B, lanes 1 and 2). Using an antibody that recognizes an epitope encoded in exon 4 of the wild-type ER-α the band corresponding to the d3/4 protein is not seen, while wild-type ER-α is still detected (Figure 4B, lanes 3 and 4). Ligand binding analysis of the *in vitro* translated d3/4 protein showed little or no ability to bind estradiol specifically (data not shown). This

protein is missing the second zinc finger of the DNA binding domain and as such would not be expected to bind to DNA. We found that under conditions in which *in vitro* transcribed/translated wild-type ER-α could bind to an oligonucleotide containing the vitellogenin B1 ERE, d3/4 did not demonstrate any specific DNA binding in gel mobility shift assays (data not shown).

To examine the transcriptional activity of d3/4 variant ER-α, transient transfections using ER negative cell lines were carried out. Under conditions in which transiently transfected wild-type ER-α was transcriptionally active and able to induce CAT activity in a ligand-dependent fashion, the d3/4 ER-α did not demonstrate any transcriptional activity on its own (Figure 6A and 6B). This is unlikely to be due to low levels of expression of this transgene, since after transfection of 5 μg of d3/4 vector into MCF10A1 cells we were able to detect a protein corresponding in size to the expected d3/4 protein (Figure 5). To determine if d3/4 ER-α and wild-type ER-α could interact to influence transcription, cotransfections of wild-type and d3/4 ER-α into MDA-MB-231 and MCF10A1 ER negative breast epithelial cell lines were carried out (Figure 6A and B). HEGO transfected alone showed the expected estrogen-dependent activity while d3/4 alone had no transcriptional activity (Figure 6A and B). However, when increasing amounts of d3/4 ER-α were transfected with a constant amount of HEGO, d3/4 could increase both the basal and estrogen-dependent activity of wild-type ER-α. When equal amounts of d3/4 and wild-type HEGO were transfected into MDA-MB-231 and MCF10A1 cells a significant increase in the estrogen-dependent activity was seen. Increasing amounts of cotransfected d3/4 was associated with statistically significant increases in basal transcription in both MCF10A1 and MDA-MB-231 cells. In MDA-MB-231 cells when 1 pmol of d3/4 was transfected with 0.5 pmol of HEGO a statistically significant increase in basal transcription was observed, while in MCF10A1 cells we saw a statistically significant increase in basal transcription when 2 pmol of d3/4 was transfected with 0.5 pmol of HEGO.

We next examined the effects of introducing the d3/4 ER-α into the parental T5 cells. Transient transfection of d3/4 into T5 cells was carried out and ER-α transcriptional

activity measured. Figure 7 shows the results obtained, and demonstrates that transfection of 1 pmol of d3/4 caused a significant increase in CAT activity both in the presence and absence of added estrogen, despite the fact that this variant ER-α does not bind appreciably to ligand *in vitro* nor has transcriptional activity of its own at this concentration (Figure 6A and 6B).

Discussion

Numerous studies have identified variant ER-α mRNAs in both normal and neoplastic breast tissue and cell lines (16,18,22-25). While still a controversial topic, evidence is emerging to support the existence of ER-α variant proteins, which could correspond to some ER-α variant mRNAs, in some cell lines and tissues *in vivo* (22,26-32). However, the pathophysiological significance of ER-α variant expression is unclear. Altered expression of some ER-α variant mRNAs was found associated with both breast tumorigenesis and breast cancer progression (16,22,33-35). Several studies, using transient transfection analyses, have shown that individual ER-α variant proteins can have both positive and negative effects on wild-type ER-α activity (22,26,27,36-40). Conflicting results for some ER-α variants have been obtained (39,41) which may be due to cell and promoter specific events previously identified for various structure/function domains of the wild-type ER-α (42,43). Similarly, overexpression of a single ER-α variant using stable transfection technology has given different results in different laboratories (36,44). Moreover, direct correlation of any single ER-α variant with clinical tamoxifen resistance or tamoxifen resistance of breast cancer cells in culture has not been forthcoming. Since most of these comparisons have been performed using individual ER-α variants and do not take into account the entire spectrum of ER-α variants relative to each other, the conclusions remain controversial. Together, the data support the hypothesis that the development of hormone-independence and endocrine resistance in human breast cancer is a multifactorial process and indeed there are many examples where the development of estrogen-independent growth and antiestrogen resistance are dissociable events in breast cancer cell line models (45-48). Similar to these and other studies, we have found that the development of estrogen-independent growth in a breast cancer cell line model, through long term growth in estrogen-depleted medium, was not associated with antiestrogen resistance. Although the estrogen-nonresponsive T5-PRF cells have a reduced sensitivity to the pure antiestrogen ICI 164,384, their growth response to 4-hydroxytamoxifen is

identical to parental T5 cells (8). However, when we investigated the relative pattern of expression of ER-α deleted variant mRNA in T5-PRF compared to parental T5 cells, there was a significant difference in the relative expression of a previously described exon 3 plus 4 deleted ER-α variant mRNA (reviewed in 49). Although the question of whether this ER-α variant is a cause of estrogen independence or merely an effect of the selection process for estrogen independence requires further study, our data, using transient transfection analyses tend to support a possible functional role for the putative 3/4 deleted ER-α protein encoded by the variant mRNA in the phenotype observed in T5-PRF human breast cancer cells. In this study we have shown that T5-PRF cells have significantly increased ligand-independent (basal) ER-α activity (reflected in both ERE-tk-CAT activity and endogenous PR levels). The d3/4 variant ER-α was able to confer increased ligand-independent (basal) and estrogen-responsive transcriptional activity when expressed in parental T5 cells and when coexpressed with wild-type ER-α in ER-α negative human breast cell lines. While the demonstrated effect of d3/4 to increase HEGO basal transcriptional activity in the ER-α negative cell lines suggests a putative functional role for this variant ER-α, this effect required 2-4 times higher levels of d3/4 than HEGO. Although such data provide 'proof of principle' that the d3/4 ER-α variant can modulate both the ligand dependent and independent transcriptional activity of wild type ER-α, the relevance of the expression levels of each protein achieved in the reconstituted transient expression system to the endogenous levels of ER-α and d3/4 ER-α variant expression in T5-PRF is unclear. Furthermore, differences in the background of transcriptional co-activators and co-repressors between naturally ER-α positive and negative cell lines (for example, 43), as well as the presence of other naturally occurring ER-α variants in naturally ER-α positive cell lines are all likely to impact on the final outcome of ER mediated transcriptional activity and underlie the differences seen between the transiently manipulated cells and the naturally occurring T5-PRF phenotype. Moreover, expression of ER-β and/or its variants may influence estrogen action (50). Both T5 and T5-PRF cells express low levels of ER-β mRNA determined by reverse transcription polymerase chain

reaction analysis (51, unpublished data), however, the functional significance of the levels remains unknown. Nonetheless, we saw a significant effect on ER-α ligand-independent transcription in T5 cells at levels of co-transfected d3/4 that likely would not be higher than the endogenous ER-α in these cells, but the extrapolation of these data to the relative expression of wild-type ER-α and d3/4 ER-α variant in T5-PRF cells is presently unknown. It is of significance that we can reproduce an effect of this d3/4 variant in the parental T5 cells, which would likely contain a more representative background of ER-α accessory proteins (ie. co-activators and/or co-repressors) as well as other variant forms of ER-α which would all contribute to the final ER mediated biological response. As well, our data do not exclude the possibility that other alterations have occurred in T5-PRF cells which, in combination with an altered ER-α variant, may contribute to the estrogen-independent phenotype of T5-PRF cells.

It has previously been shown that breast cancer cells can adapt to low levels of estrogen by enhancing their sensitivity to estrogen (52). Estrogen-deprivation of MCF-7 human breast cancer cells resulted in estrogen hypersensitivity and maximal growth was achieved with an estrogen concentration 4-5 orders of magnitude lower than wild-type cells. These researchers also found that the concentration of ICI needed to inhibit growth of these cells was ~6 orders of magnitude lower than wild-type cells, supporting the hypothesis in this model, that increased sensitivity to ER ligands had occurred. While supersensitivity to estrogen in T5-PRF cells cannot be entirely ruled out, we have previously shown that, while T5-PRF cells are sensitive to growth inhibition by ICI 164,384, in contrast to the data of Masamura *et al* , they are less sensitive than the parental T5 cells (i.e. ID_{50} 100 nM and 5μM for T5 and T5-PRF, respectively), suggesting that in this model other mechanisms are likely involved.

Our data do not address the mechanism by which d3/4 enhances ER transcriptional activity, but several possibilities exist. The ER-α contains at least two separate regions that are required for optimal transcriptional activation (42,43). The amino-terminal region contains promoter and cell-type specific ligand-independent transcriptional activity (AF1)

and the second, AF2, is located in the ligand-binding carboxyl-terminus of the receptor. Exon 3/4 deleted ER-α containing an intact AF2 or AF1 domain could interfere with, or sequester an ER-α repressor protein resulting in increased ER-α transcriptional activity in the absence of ligand (53). This variant may also retain the ability to interact with other ER-α regulatory proteins such as coactivators or components of the basal transcription machinery.

ER-α also contains two domains involved in dimerization (54,55). A weak dimerization interface is present in the DNA-binding domain (DBD) and a strong interface is located in the C-terminal ligand-binding domain (56). d3/4 containing an intact C-terminal dimerization domain, may form heterodimers with wild-type ER-α that have altered transcriptional regulatory properties through differing protein-protein interactions.

The crystal structure of the ER-α hormone binding domain has recently been elucidated (57). Based on this structure, the d3/4 protein would contain many of the regions essential for transactivation, including the predominant helix 12 (encompassing amino acids 539-547). However, since d3/4 alone has no transcriptional activity (at least on a classical ERE regulated promoter) the structure must be sufficiently altered to prevent activity, or AF2 can only be activated in a ligand-dependent manner but d3/4 cannot bind ligand. Helix 12 in AF2 is believed to be the main region involved in coactivator recruitment and it may be possible that d3/4, following heterodimerization with ER-α, enhances recruitment of coactivators to the basal transcription complex and this enhances ER-α activity.

We have found that using a transient expression system, the d3/4 ER-α caused increased ligand-independent wild-type ER-α activity and also enhanced the ligand-induced ER-α transcriptional activity, despite the fact that on its own this variant is not transcriptionally active on a classical ERE promoter, nor does it bind ligand *in vitro* to any significant degree. Studies have demonstrated that the ability of steroid hormone receptors to modulate transcription does not necessarily require that the receptors bind DNA. PRc, an N-terminally truncated PR isoform lacking the first zinc-finger of the DBD, has no

transcriptional activity of its own but has been shown to enhance progestin-induced transcriptional activity (58). The DBD of the ER-α does not appear to be necessary for raloxifene activation of the TGFβ3 gene (59) and ER-α can activate transcription from AP-1 dependent promoters through a DNA binding-independent pathway (60). Sp1 and ER-α directly interact to enhance estrogen-induced transactivation of the Sp1-dependent Hsp27 gene promoter and the DBD of the ER-α is not required (61).

Recent research has demonstrated that the ER-α can be activated in a ligand-independent fashion (62-64). Studies have shown that several growth factors such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and insulin-like growth factor (IGF-1) were able to activate the ER-α in the absence of ligand. The ability to activate the ER-α in the absence of estrogen could confer a growth advantage to breast cancer cells and aid in the development of a hormone-independent phenotype. The presence of alternate forms of ER-α capable of interacting with wild-type ER-α to increase ligand-independent activity could also confer a potential growth advantage to breast cancer cells. A recent study has shown that constitutively active, ligand-independent ER-α mutants undergo conformational changes and interactions with coactivators that mimic changes in ER-α that are usually regulated by ligand (65). Recently, researchers have shown that TR-β2 is a ligand-independent activator of the gene encoding thyrotropin-releasing hormone (TRH) and have mapped a region in the N-terminus of the receptor responsible for this activity (66). These researchers suggest that the mechanism of ligand-independent activation involves direct interaction of the TR-β2 amino terminus with either transcriptional cofactors or the basal transcription machinery itself.

An increased relative expression of variant ER-α proteins containing intact AF domains, could result in increased interactions with the ER-α and/or other proteins involved in ER-α transcriptional activity. This could be a potential mechanism for estrogen-independent growth associated with the presence of one or more variant ER species and could explain the increased ER-α activity we have seen with the d3/4 ER-α.

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FIGURE LEGENDS

Figure 1. ER transcriptional activity. A. T5 and T5-PRF cells were transfected and CAT assays performed as described in Methods. Results are expressed as fold CAT activity compared to T5 basal (arbitrarily set at 1.0). *$p<0.05$, Student's t-test (compared to T5 basal). Results represent mean ± SEM, n=7. **B.** T5 and T5-PRF cells were transfected and CAT assays performed as described in Methods. T5-PRF cells were treated with ICI 164,384 alone under basal (estrogen-free) conditions. Results represent fold CAT activity as compared to T5 basal, n=2.

Figure 2. Basal progesterone receptor levels. PR levels were determined by whole cell binding as described in Methods. PR levels are expressed as fmol PR/10^6 cells and results represent mean ± SEM, n=3, **$p<0.01$, Student's t-test.

Figure 3. Identification of exon 3/4 deleted variant ER-α mRNA. A. Long-range ER-α RT-PCR. Total RNA was extracted from T5 and T5-PRF cells, reverse transcribed and PCR amplified using 1/8U and 1/8L primers. Labelled PCR products were separated on 3.5% acrylamide-urea gels and visualized using autoradiography. WT-ER = wild type ER-α, D7-ER = exon 7 deleted ER-α, D4-ER = exon 4 deleted ER-α and D3/4-ER = exon 3 and 4 deleted ER-α, based on size compared to labelled markers (not shown). **B.** Sequence of d3/4 ER-α cDNA. The 928bp PCR product was excised from a gel and subcloned (in triplicate) and three colonies from each independent subcloning were sequenced to confirm the identity of d3/4 cDNA.

Figure 4. ***In vitro*** **transcription/translation and Western blotting of d3/4 protein. A.** Expression vectors containing wild-type ER-α (lane 1) and d3/4 cloned into pSG5 (lane 2) were subjected to *in vitro* transcription/translation reactions in the presence of ^{35}S-methionine. Reaction products were run on 10% SDS-acrylamide gel, dried and exposed to film overnight. **B.** *In vitro* transcription/translation products were run on 10% SDS-acrylamide gels, transferred to nitrocellulose and Western blotting performed. Lanes 1 and 2 were visualized

using ER-α Ab H226 (epitope located in ER-α exon 1/2) and lanes 3 and 4 using ER-α Ab 308 (epitope located in ER-α exon4).

Figure 5. ***In vivo*** **expression of d3/4 protein.** MCF10A1 human breast epithelial cells were transfected with the appropriate expression vector, cells were lysed in 8M urea, and 10 μg protein run on a 10% SDS-acrylamide gel. Gels were transferred to nitrocellulose and Western blotting performed using ER-α Ab H226. Lane 1. Control cells transfected with 5 μg of vector (pSG5) alone; Lane 2. Cells transfected with 5 μg HEGO (WT-ER-α) expression vector; Lane 3. Cells transfected with 5 μg d3/4 expression vector; Lane 4. *In vitro* transcribed/translated WT-ER-α (1 μl); Lane 5. *In vitro* transcribed/translated d3/4 ER-α (2 μl).

Figure 6. Activity of d3/4 in ER-α negative cells. A. MDA-MB-231 cells were transfected with 5μg ERE-tk-CAT, 1μg pCH110, 0.5pmol HEGO ± 0.5-2pmol d3/4 ± vector DNA to give a total of 17 μg DNA/dish . Cells were treated with 10nM estradiol (E2) for 24h or vehicle alone as control. Results are expressed as fold CAT activity compared to basal HEGO activity arbitrarily set as 1. Histograms represent mean ± SEM, n=5-7. *b=$p<0.05$ paired Student's. t-test, result compared to basal HEGO alone; *e=$p<0.05$ paired Student's t-test, result compared to estradiol treated HEGO alone. **B**. MCF10A1 cells were similarily transfected. Histograms represent mean ± SEM, n=4. *b=$p<0.05$ paired Student's t-test, result compared to basal HEGO alone, *e=$p<0.05$, paired Student's t-test, result compared to estradiol treated HEGO alone.

Figure 7. Transient transfection of d3/4 expression vector into T5 cells. Cells were grown in PRF-DMEM as described in Methods and transfected with 5μg ERE-tk-CAT expression vector, 5 μg pCH110 along with the appropriate amount of d3/4 expression vector. Cells were treated with vehicle or 10nM estradiol (E2) for 24h, harvested and CAT assays performed. Results represent mean ± SEM, n=2-5, *b=$p<0.05$ Wilcoxon's rank sum test, result compared to basal ERE-tk-CAT activity, *e=$p<0.05$ Wilcoxon's rank sum compared to estradiol treated ERE-tk-CAT activity.

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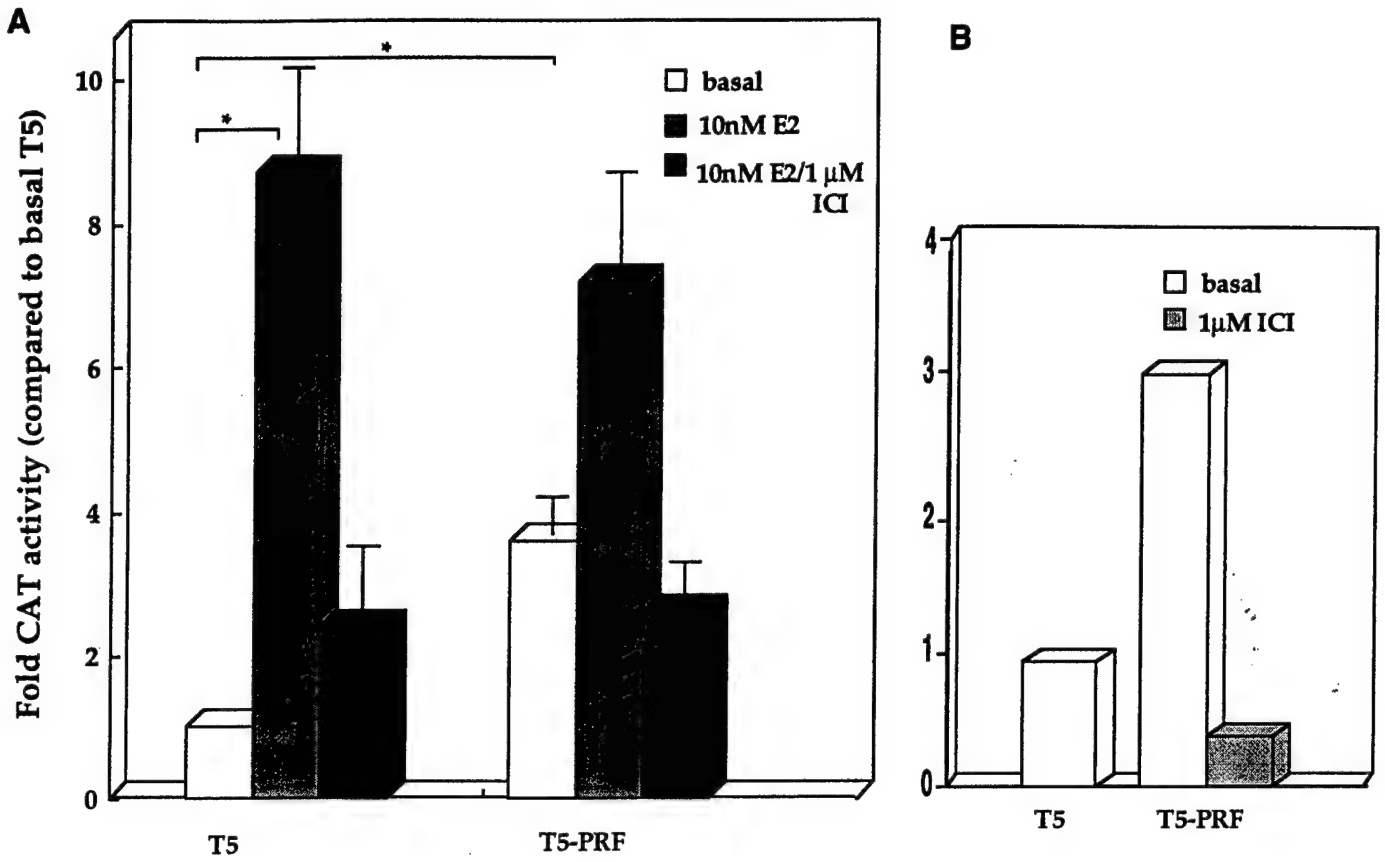
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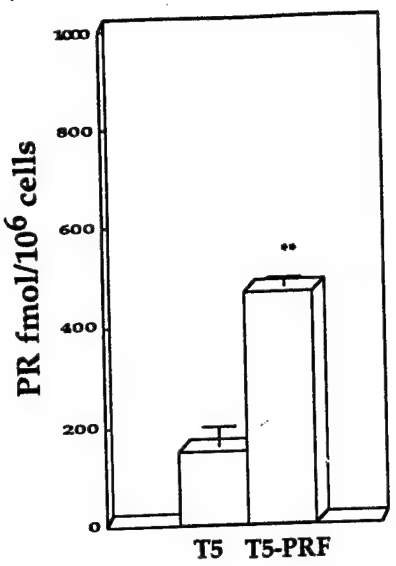
Figure 1



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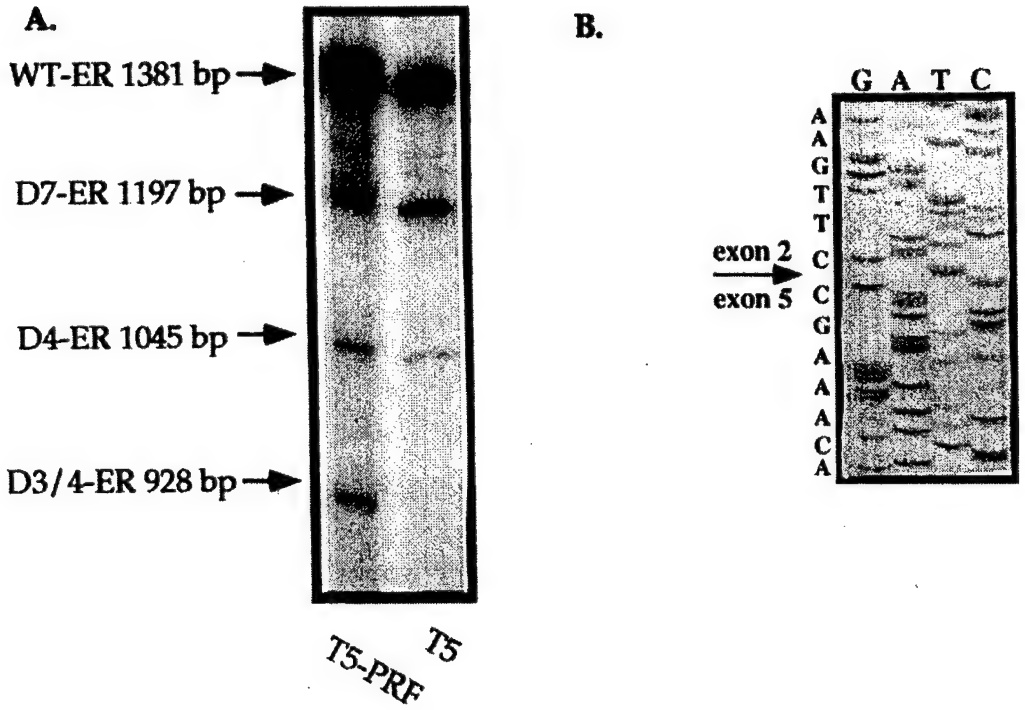
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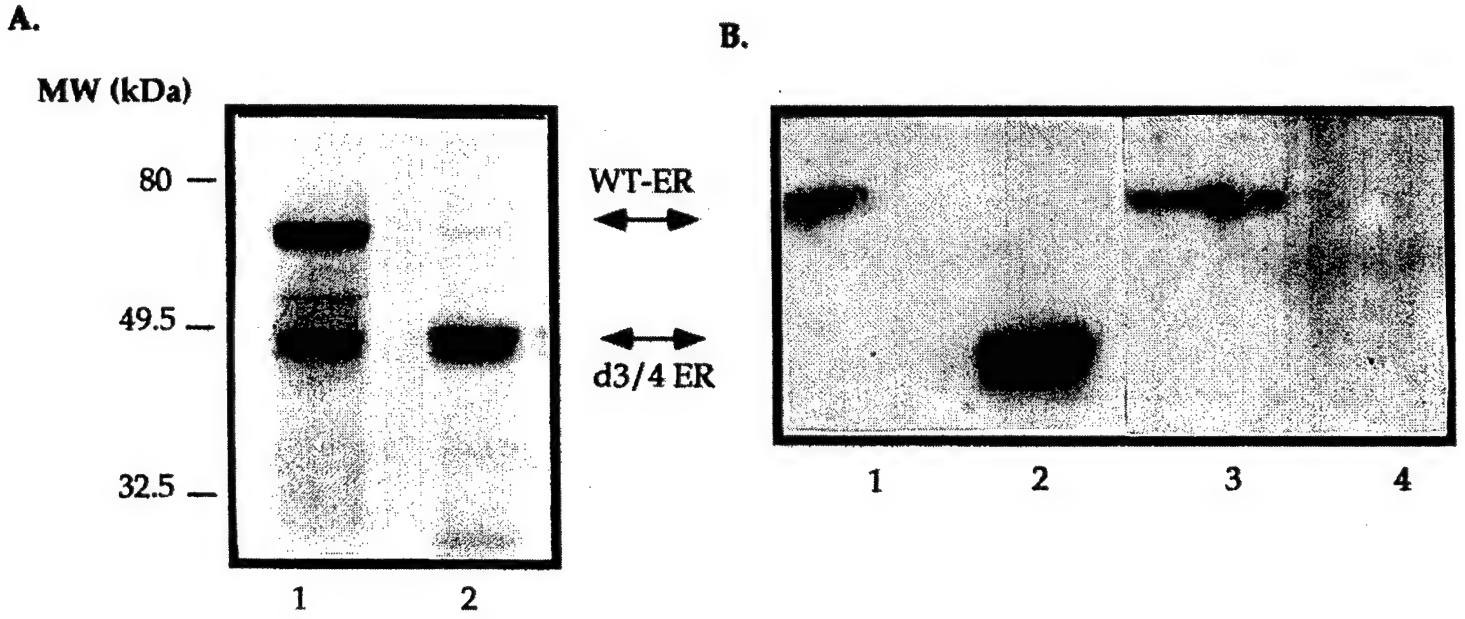
Fig 3



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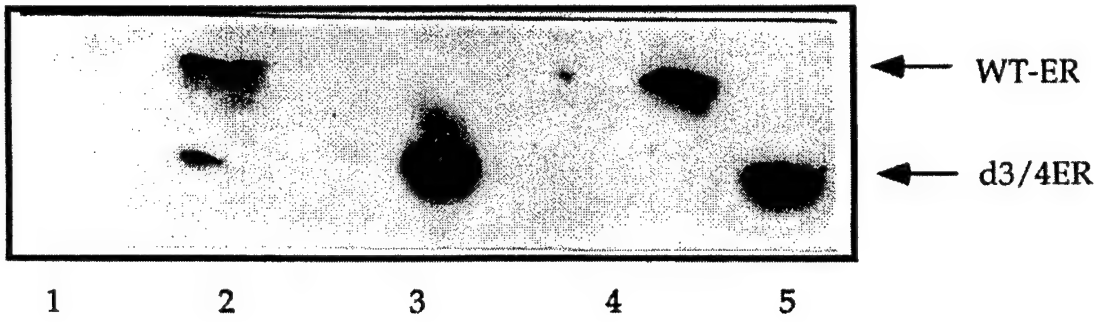

Fig 5

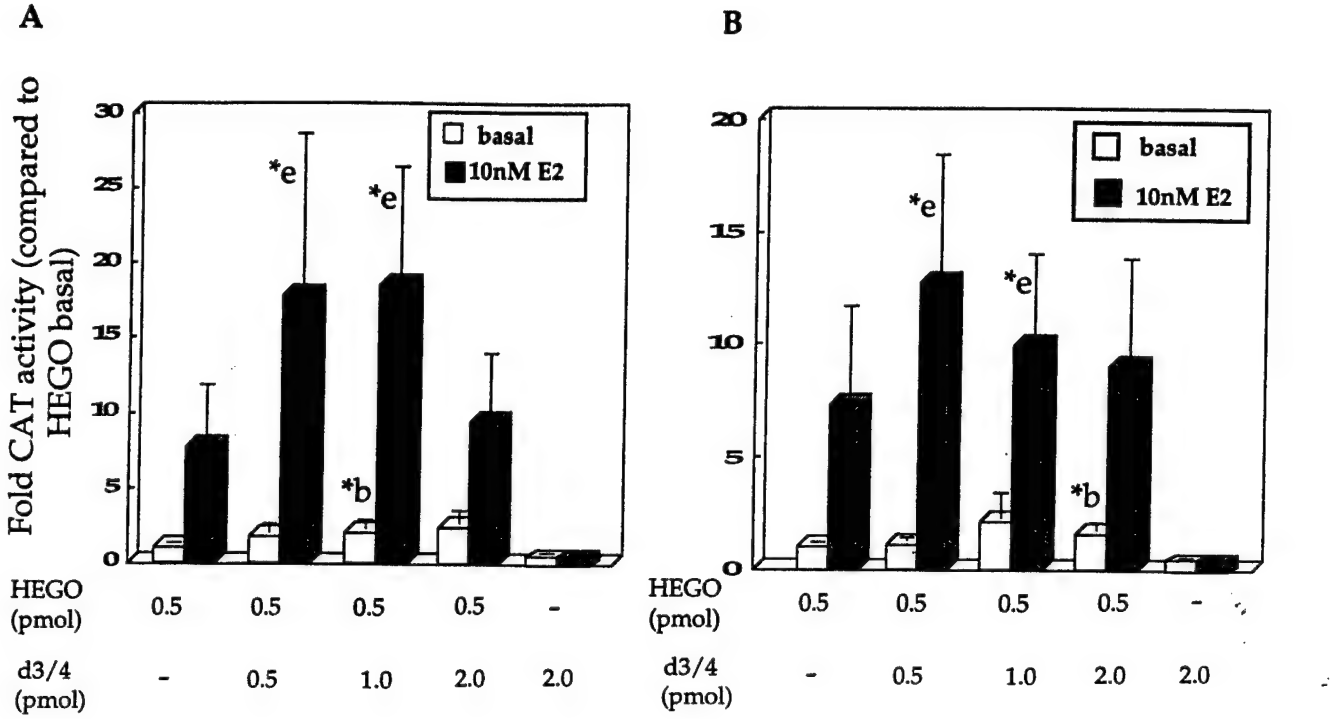
A
B
Fold CAT activity (compared to HEGO basal)
basal
10nM E2
*e
*b
HEGO (pmol)
d3/4 (pmol)
0.5
-
1.0
2.0

Figure 6.

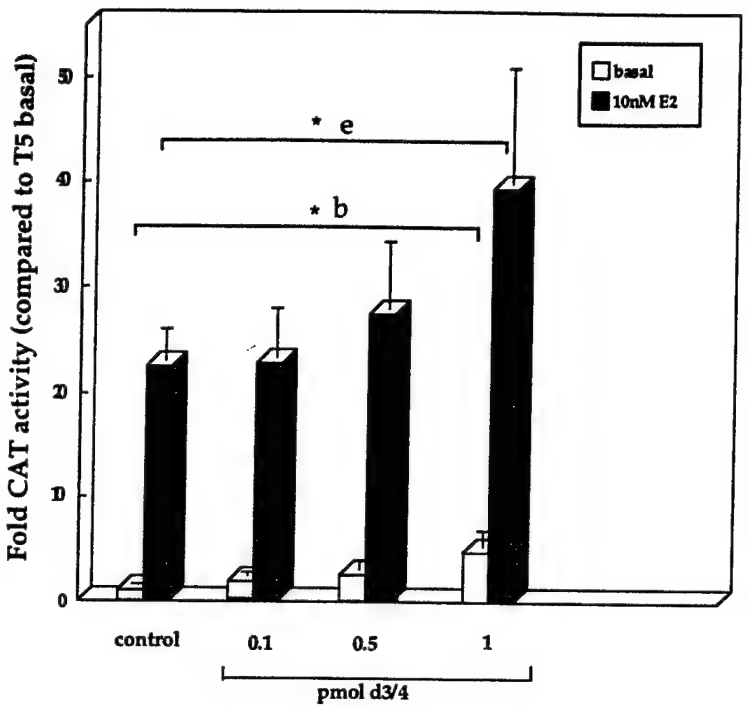
basal
10nM E2
* e
* b
Fold CAT activity (compared to T5 basal)
control
0.1
0.5
1
pmol d3/4

Figure 7.

APPENDIX 5

1997, Breast Cancer Res. Treat., 46, 48.

173 TAMOXIFEN DOWN REGULATES PHORBOL 12-MYRISTATE 13-ACETATE INDUCED NITRIC OXIDE PRODUCTION IN ZR-75-1 HUMAN BREAST CANCER CELLS. Alalami O* & Martin JHJ Division of Biomedical Sciences, School of Health Sciences, University of Wolverhampton, Wolverhampton, England, WV1 1DJ.

The purpose of this study was to determine if the L-arginine/NO pathway was present in ZR-75-1 human breast cancer cells and to investigate possible effects of tamoxifen.

ZR-75-1 cells ($5x10^4$ cells/ml) were cultured in the presence and absence of various drugs. After 2 days *in vitro* the NO_2^- concentration in conditioned medium was quantitated by the Greiss reaction.

Treatment of ZR-75-1 cells with L-arginine (10mM) increased nitrite production from 2nmol to 70nmol ($p<0.001$). This could be significantly ($p<0.001$) inhibited by L-NAME (2mM). Treatment with phorbol 12-myristate 13-acetate (PMA) (200nM-1000nM) caused a significant increase ($p<0.001$) in NO_2^- secreted into the culture medium. Although tamoxifen (10^{-8}M) had no effect on control levels of NO_2^- production, tamoxifen was able to significantly ($p<0.001$) down-regulate PMA enhanced nitrite production by ZR-75-1 cells.

We conclude that tamoxifen can down regulate PMA induced NO production by ZR-75-1 human breast cancer cells. As nitric oxide has been implicated in several areas of tumour biology including metastasis, differentiation and angiogenesis we suggest that further investigations of the effects of anti-estrogens on this pathway would be of value.

174 CORRELATION BETWEEN GCDFP-15 AND HORMONE RECEPTORS IN HUMAN BREAST CARCINOMA. Wheeler HJ*, Moe RE, Gown AM, University of Washington Medical Center, Seattle, Washington 98195.

Breast carcinomas are known to be influenced by hormones, particularly estrogens and progestins. Androgen manipulation is used much less often to treat breast cancer. On the other hand, some breast cancers not only respond to androgens but progress instead of regressing with androgen therapy. A glycoprotein in breast cyst fluid, gross cystic disease fluid protein (GCDFP-15), appears in these cancers and in the peripheral blood of patients with these androgen-sensitive cancers.

This study tests the hypothesis that GCDFP-15 expression correlates with androgen receptor expression, but does not correlate with non-androgen hormone receptor expression. Correlation between expression of GCDFP-15, androgen, estrogen, and progesterone receptors was evaluated. Immunocytochemistry studies were performed on a series of 50 human breast tissue specimens, 48 with breast carcinomas and two with benign breast disease. Specimens were scored in a semi-quantitative fashion based on distribution of positive cells within the breast tumor or benign tissue, followed by a chi-square statistical analysis.

A strong correlation was found between GCDFP-15 expression and androgen receptor expression in these breast cancers ($P = 0.02$). Estrogen receptor expression in these cancer specimens was correlated with androgen receptor expression ($P = 0.002$), but the former was not significantly associated with GCDFP-15 expression ($P = 0.35$). These results support the finding of a unique subset of breast cancers correlated with GCDFP-15 and potentially stimulated by androgens. In women with cancers positive for GCDFP-15 and androgen receptors, androgen antagonists such as Flutamide might be beneficial.

175 EXPRESSION OF ESTROGEN RECEPTOR-BETA VARIANT mRNAs IN HUMAN BREAST TUMORS. Leygue E*, Dotzlaw H, Hare H., Watson PH*, Murphy LC. Department of Biochemistry and Molecular Biology and Department of Pathology*, University of Manitoba, Winnipeg, Canada, R3EOW3.

The presence of estrogen receptor-beta (ER-β) mRNA within human breast tumors and breast cancer cell lines has recently been reported (Dotzlaw et al. J Clin Endocrinol Metabolism, in press). Because several exon-deleted variant forms of the classic estrogen receptor (ER-α) have been described and are suspected to be involved in the acquisition of the hormone-independent phenotype during breast tumorigenesis, it was our aim to determine whether ER-β variant mRNAs might be expressed in human breast tumor specimens. To address this issue, a recently described RT-PCR approach (Cancer Res. 56:4324, 1996), based on the co-amplification of wild-type ER-β and all possible variant mRNAs containing putative exon 1 and exon 8 of the receptor, whose precise exonic structure is not yet known, has been used. Several PCR products were obtained in both breast tumor samples and breast cancer cell lines. The sequence of one of these bands, co-amplified with wild-type ER-β in several tumor samples and breast cancer cell lines, revealed a deletion of 273 bases that by analogy to the ER-α exonic structure, would correspond to the deletion of putative exons 5 and 6. This deleted ER-β variant mRNA, if translated, would encode an ER-β like protein truncated in 91 amino acids within the region containing the hormone binding domain and the transactivating function 2 (AF-2) of the ER-β protein. These results suggest that ER-β variants exist, and as suggested for ER-α variants might also be involved in mechanisms underlying tumor progression.

176 DISSOCIATION BETWEEN STEROID RECEPTOR EXPRESSION AND CELL PROLIFERATION IN THE NORMAL HUMAN MAMMARY GLAND.
Clarke RB*, Howell A† and Anderson E. Clinical Research Department and †CRC Department of Medical Oncology, (University of Manchester), Christie Hospital (NHS) Trust, Wilmslow Road, Manchester, M20 4BX, UK.

Oestradiol (E_2) stimulates cell proliferation and progesterone receptor (PgR) synthesis in luminal epithelial cells of the normal breast[1], presumably acting via the specific nuclear E_2 receptor (ER). Approximately 10% of epithelial cells within the breast express immuno-detectable ER but little is known about their distribution and their organisation in relationship to proliferating cells and those expressing the PgR. We performed double labelling on breast tissue sections by an indirect peroxidase method using antibodies to ER or PgR and ^{3}H-thymidine (^{3}H-d[T]) histo-autoradiography, and also by immuno-fluorescence using antibodies to steroid receptors and the Ki67 proliferation antigen. The results from normal human breast tissue indicate that ER-positive cells are distributed evenly throughout the mammary epithelium. These cells also synthesise the PgR but are rarely observed to divide (see table). Conversely, in ER-positive human mammary tumours, a significantly higher proportion of dividing cells express ER (22.4%; range 0-84%). These data indicate that normal breast epithelial cells capable of proliferation do not express ER and may be stimulated by E_2 indirectly via secretion of paracrine factors. In some tumours, however, cells may have acquired proliferative capacity and be directly stimulated by E_2.

	Number of Samples	Total Cells	ER+ve (%)	PgR+ve (%)	^{3}H-d[T] (%)	Ki67 (%)	Receptor+ve and Proliferative (%)
Normal	10	10,026	1735 (17.3)	-	46 (0.5)	-	1 (2)
Normal	25	25,302	-	3232 (12.8)	382 (1.5)	-	17 (4.7)
Normal	25	28,395	2107 (7.4)	-	-	639 (2.3)	9 (1.3)
Normal	25	28,018	-	3231 (11.5)	-	391 (1.4)	7 (1.4)
Normal	13	13,895	1792 (12.9)	1765 (12.7)	-	-	1727 (96*)
Tumour	19	21,245	6693 (31.5)	-	-	1235 (5.8)	275 (22.4)

1 Laidlaw *et al.* (1995) Endocrinol. **136**, 164-171. *Receptors coexpressed

APPENDIX 6

1998, Mol. Cell. Endocrinol., 138, 199.

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Molecular and Cellular Endocrinology 138 (1998) 199–203

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Molecular and Cellular Endocrinology

Rapid communication

Estrogen receptor-β mRNA variants in human and murine tissues

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Abstract

Estrogen receptor (ER)-β mRNA splice variants have been identified in human breast tumors as well as normal human and mouse ovarian, uterine and mammary tissues. In both species transcripts deleted in exons 5 or 6, or 5 + 6 have been characterized by RT-PCR followed by cloning and sequencing. In mouse tissues an ER-β transcript containing 54 nucleotides inserted in frame between exons 5 and 6 was identified. Interestingly, no equivalent of the mouse inserted transcript was detected in any of the four human tissues analyzed.

Keywords: Estrogen receptor-α; Estrogen receptor-β; mRNA variant; Alternative splicing; Human; Mouse

1. Introduction

Recently, the cDNA of a second estrogen receptor, estrogen receptor (ER)-β, was cloned and sequenced from the rat (Kuiper et al., 1996), the human (Mosselman et al., 1996) and the mouse (Tremblay et al., 1997). Northern analysis of RNA isolated from mouse ovary demonstrated the presence of multiple mRNA species for ER-β (Tremblay et al., 1997) suggesting the possibility that variant ER-β proteins might exist. To investigate whether ER-β variant mRNAs might be expressed in human as well as murine tissues, an RT-PCR analysis was undertaken which demonstrated the presence of variant ER-β mRNAs in both species.

2. Materials and methods

2.1. Tissues and RNA extraction

Human breast tumor specimens left-over from steroid receptor assays were obtained from the Manitoba Breast Tumor Bank, and three non-malignant human uterine hysterectomy samples were obtained from the Department of Obstetrics and Gynecology (Health Sciences Centre, Winnipeg, Canada). Total RNA was extracted by the guanidinium thiocyanate/cesium chloride method as previously described (Dotzlaw et al., 1990). Four nonmalignant human ovarian samples from two pre-menopausal and two post-menopausal women were obtained through the Ovarian Tissue Bank (Institut du Cancer de Montreal, Centre de Recherche Louis-Charles Simard, Montreal, Canada). Four normal human breast tissues from reduction mammoplasties of pre-menopausal women were obtained through the Manitoba Breast Tumor Bank. Total RNA from the ovarian and normal

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breast tissue samples was extracted with Trizol reagent (Gibco/BRL) according to the manufacturer's instructions.

Mouse uteri and ovaries were obtained from four female mice aged 8–9 weeks, and mammary tissues were obtained from two adult lactating female mice. Total RNA was extracted with Trizol reagent (Gibco/BRL) according to the manufacturer's instructions.

Integrity of RNA was confirmed by denaturing gel electrophoresis as previously described (Murphy and Dotzlaw, 1989).

2.2. RT-PCR and primers

Total RNA (1.5 μg per reaction) was reverse transcribed as previously described (Dotzlaw et al., 1997). One microlitre of this reaction was amplified by PCR incorporating ^{32}P in a final volume of 10 μl, and 4 μl of this reaction separated on 6% denaturing polyacrylamide gels and autoradiographed as previously described (Dotzlaw et al., 1997).

All ER-β exons are defined in this report by analogy to the human ER-β exon structure (Enmark et al., 1997): human primer set one: hER-β exons 4 and 7: hER-β-4 (sense) 5′-GGC CGA CAA GGA GTT GGT A-3′ (priming site in exon 4, nucleotides 762—780 as numbered in Mosselman et al. (1996)); hER-β-7 (antisense) 5′-TCC ATG CCC TTG TTA CTC G-3′ (priming site in exon 7, nucleotides 1262–1280). The PCR conditions were 30 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C.

Human primer set two: hER-β exons 5 and 6: hER-β-5 (sense) 5′-GCT GTT GGA TGG AGG TGT TA-3′ (priming site in exon 5, nucleotides 857–876); hER-β-6 (antisense) 5′-CTT GAA GTA GTT GCC AGG AG-3′ (priming site in exon 6, nucleotides 997–1016). The PCR conditions were 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C.

Mouse primer set one: mER-β exons 4 and 8: mER-β-4 (sense) 5′-CTG AAC AAA GCC AAG AGA-3′ (priming site in exon 4, nucleotides 600–617 as numbered in Tremblay et al. (1997)); mER-β-8 (antisense) 5′-GCT CTT ACT GTC CTC TGT CG-3′ (priming site in exon 8, nucleotides 1417–1436). The PCR conditions were 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C.

Mouse primer set two: mER-β exons 5 and 6: mER-β-5 (sense) 5′-GCT GAT GGT GGG GCT GAT GT-3′ (priming site in exon 5, nucleotides 890–909); mER-β-6 (antisense) 5′-ATG CCA AAG ATT TCC AGA AT-3′ (priming site in exon 6, nucleotides 993–1012). The PCR conditions were 35 cycles of 1 min at 94°C, and 30 s at 60°C.

PCR products from human breast tumors and mouse mammary tissues were subcloned into the cloning vector, pGEM-T Easy (Promega) following the manufacturer's instructions. Double stranded DNA from at least two independent clones from each tissue was sequenced with a T7 Sequencing kit (Pharmacia) following the manufacturer's protocol. All RT-PCRs were carried out at least 2 × for each sample analyzed.

3. Results and discussion

Previously the presence of ER-β mRNA was identified in some human breast tumor samples (Dotzlaw et al., 1997). The RT-PCR analysis employed a primer set which annealed to sequences corresponding to exons 7 and 8 of the human ER-β cDNA (Enmark et al., 1997). Numerous splicing variants of the human ER-α mRNA have been identified to date (Murphy et al., 1997), and it was of interest to determine if similar splice variants could be detected in the ligand binding domain of the ER-β mRNA in human breast tumors. Using a primer set which would anneal to sequences located in exons 4 and 7 of the human ER-β cDNA (Enmark et al., 1997), RT-PCR analyses were undertaken using RNA isolated from four separate human breast tumor samples which had previously been shown to express ER-β mRNA by RT-PCR using an exon 7/8 primer set (Dotzlaw et al., 1997). The results presented in Fig. 1 show the presence of the expected 519 bp wild-type ER-β product, as well as several smaller sized PCR products.

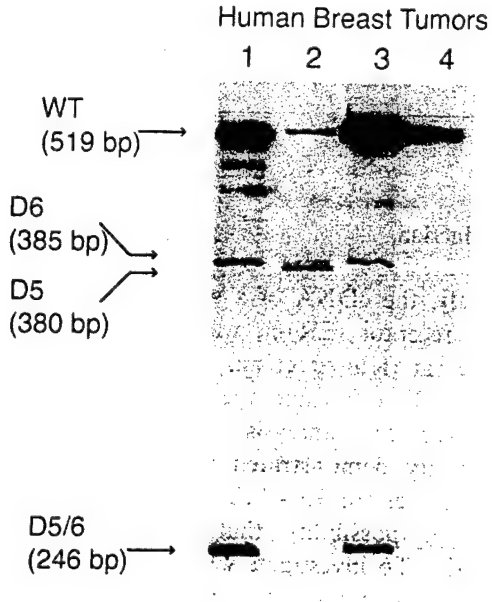

Fig. 1. Detection of wild-type ER-β and ER-β variant mRNAs in human breast tumor tissues. Total RNA extracted from four different human breast tumors (1–4) was reverse transcribed and PCR amplification was carried out using primers located in exons 4 and 7. PCR products migrating at the sizes of 519, 385, 380 and 246 bp were subsequently cloned, sequenced and identified as corresponding to ER-β wild-type (WT), exon 6-deleted variant (D6), exon 5-deleted variant (D5) and exon 5–6-deleted variant (D5/6) cDNAs, respectively.

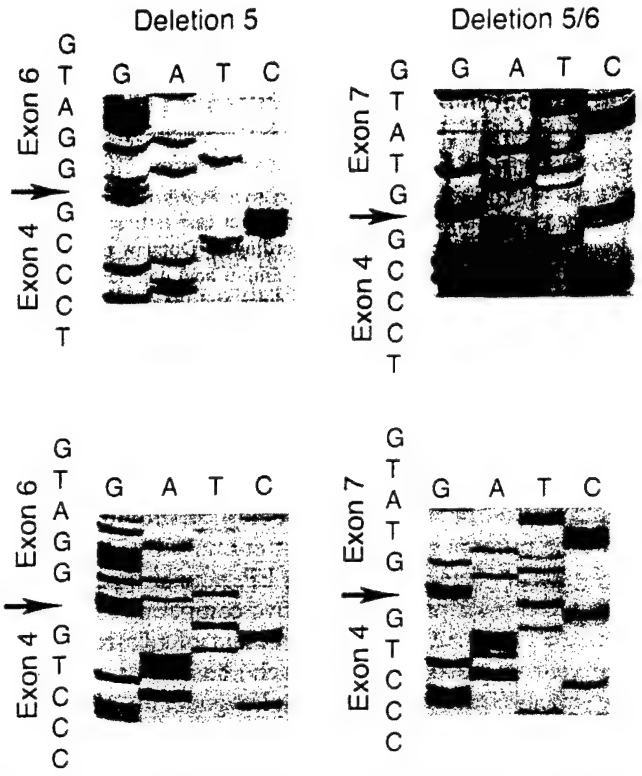

Fig. 2. Sequencing of exon 5-deleted and exon 5/6-deleted ER-β variants in human and murine tissues. Top panels: sequencing of PCR products obtained by amplification of human breast tumor cDNAs using primers in exons 4 and 7 and migrating at the sizes of 380 and 246 bp (Fig. 1) showed a perfect junction between exon 4 and 6 (deletion 5), and exon 4 and 7 (deletion 5/6), respectively. Bottom panels: sequencing of PCR products obtained by amplification of murine breast tissue cDNAs using primers in exons 4 and 8 and migrating at the sizes of 698 and 564 bp (Fig. 3) showed a perfect junction between exon 4 and 6 (deletion 5), and exon 4 and 7 (deletion 5/6), respectively.

Cloning and sequencing of the smaller sized products (Fig. 2) revealed deletions of nucleotides 812–950, 951–1084 and 812–1084 (numbered as in Mosselman et al. (1996)) which are precise exon deletions of exon 5, 6, and 5 + 6, respectively. To determine if such deletions occurred only in human breast tumor tissue, RNA extracted from several normal breast, uterine and ovarian tissue samples was analyzed (Fig. 3, top panel). The same tissues from the mouse were analyzed in parallel, with mouse ER-β primer set located in putative exons 4 and 8 (Fig. 3, bottom panel; all exons, mouse and human, are numbered according to the human ER-β structure (Enmark et al., 1997)). All human tissues analyzed expressed ER-β variant mRNAs similar to those identified in breast tumors. The expected wild-type product of 837 bp was detected in all mouse tissues, as were several smaller sized PCR products. Cloning and sequencing of the 698 and 564 bp fragments identified deletions of nucleotides 829–967 and 829–1101, which by analogy to the human ER-β would be a precise deletion of exon 5 and deletion of exons 5 and 6, respectively (Fig. 2, nucleotides numbered as in Tremblay et al. (1997)). The 703 bp band was found to correspond to an exon 6 deleted ER-β transcript.

These data are the first to support the expression of exon deleted splice variants for ER-β similar to those for ER-α, in both human and murine tissue samples. The exon 5 and 6 deleted splice variants identified in this

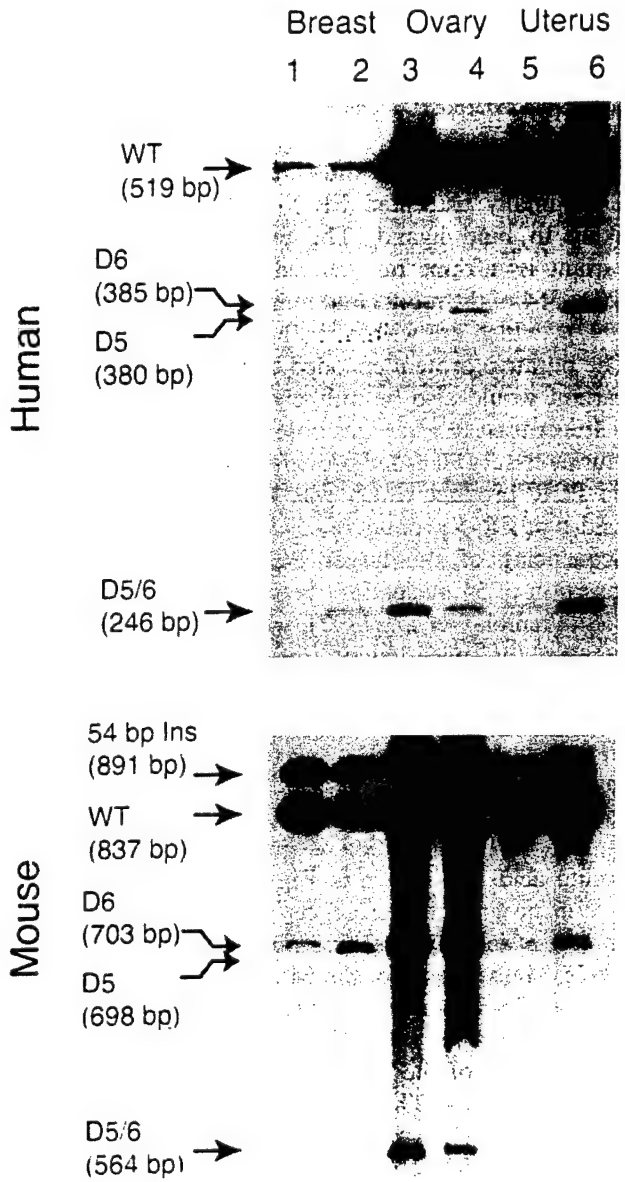

Fig. 3. Detection of wild-type ER-β and ER-β variant mRNAs in normal human and murine tissues. Total RNA extracted from normal human (top panel) or mouse (bottom panel) breast tissues (1–2), ovaries (3–4) and uteri (5–6) was reverse transcribed and PCR amplification was carried out using primers located in exons 4 and 7 (human) or in exons 4 and 8 (mouse). PCR products obtained in human tissues migrated at the sizes of 519, 385, 380 and 246 bp corresponding to ER-β wild-type (WT), exon 6-deleted (D6), exon 5-deleted (D5) and exon 5–6-deleted (D5/6) cDNAs, respectively. PCR products obtained in mouse tissues and migrating at the sizes of 891, 837, 703, 698 and 564 bp were identified as corresponding to a 54 bp inserted ER-β variant (54 bp Ins), ER-β wild-type (WT), exon 6-deleted variant (D6), exon 5-deleted variant (D5) and exon 5–6-deleted variant (D5/6), respectively.

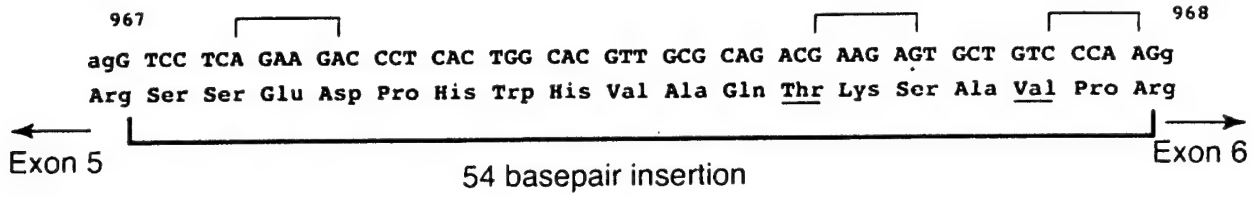

Fig. 4. Insertion (54 bp): nucleotide and amino acid sequences. The sequence of the inserted 54 bp is indicated in upper case letters. Nucleotides corresponding to the published mouse ER-β cDNA sequence, indicated in lower case letter, are numbered according to Dotzlaw et al. (1990). Brackets indicate putative exonic splicing enhancer sequences (Coulter et al., 1997; Otto et al., 1997). Predicted amino acid composition of the insert is shown. The underlined amino acids correspond to the substitutions observed between mouse inserted sequence and the recently described rat inserted sequence (Daffada et al., 1994).

study are out-of-frame and would be expected to encode C-terminally truncated ER-β proteins, which are unlikely to bind ligand. The exon 5 + 6 deleted splice variant is inframe but deleted in 91 amino acids (aa) (274–364 of the mouse and human ER-β (Enmark et al., 1997)), which are within the hormone-binding domain. The putative protein encoded by the exon 5 + 6 deleted variant would also be unlikely to bind ligand.

In contrast to what was observed in human tissues, all murine tissues analyzed presented a prominent ER-β 891 bp PCR product which was larger than the expected wild-type ER-β product of 837 bp (Fig. 3, bottom panel). Sequencing of the larger PCR product revealed an insertion of 54 nucleotides between nucleotides 967 and 968 (Tremblay et al., 1997), which is precisely inserted between the splice junction of exons 5 and 6. Identical results were obtained when the starting RNA samples were enriched for polyadenylated transcripts using oligo-dT attached to magnetic beads (data not shown), suggesting that the inserted transcript represented an authentic mRNA species. The sequence of this insertion is shown in Fig. 4. This insertion is inframe and the predicted amino acids are shown in Fig. 4 also. While this work was in progress the presence of a 54 bp inserted ER-β transcript in rat tissues was published (Chu and Fuller, 1997). The sequence of the 54 nucleotide insertion in the murine ER-β transcript is identical to that published for the rat except for a T → C change at nucleotide position 36 (1 = start of the 54 nucleotide insert), which would result in a Met → Thr substitution in the mouse protein, and a C → T change at position 48 which would result in an Ala → Val substitution in the mouse protein.

Because the initial screening of human tissues using the exon 4/7 primer set failed to reveal an analogous human ER-β transcript containing an insertion between exons 5 and 6, reanalysis of human and mouse tissues was undertaken using primer sets located in exons 5 and 6 of either the human or the mouse ER-β. While the inserted ER-β transcript was easily detected as a 177 bp PCR product in all murine tissues analyzed, only the expected 160 bp PCR product corresponding to the human wild-type ER-β mRNA was detected in the normal human tissues (Fig. 5), and an inserted ER-β variant was similarly not detected in ten human ER-β RNA positive breast tumor samples (Dotzlaw et al., 1997) (data not shown). The data shown in Fig. 5 suggest that the inserted transcript is predominant in both mouse mammary gland and uterus, while similar levels of each transcript occur in the mouse ovary. It should be noted that the primers used in this latter analysis (Fig. 5) were designed to detect the mouse ER-β wild-type and the inserted transcript. Thus only two competing PCR products are obtained, and measurement of relative expression using such an approach has been validated previously (Daffada et al., 1994; Leygue et al., 1996). This is in contrast to the primer set used in Fig. 3 which detects up to five PCR products, is designed for the study of relative patterns of expression and is unlikely to accurately reflect the relative expression of any two individual species under such conditions.

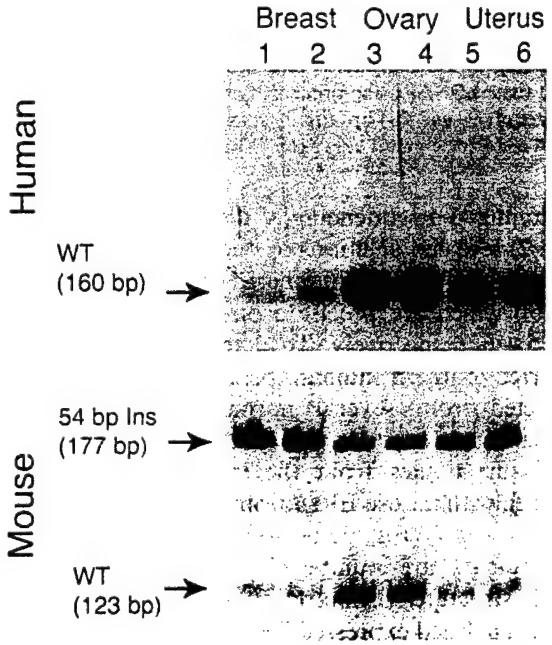

Fig. 5. Amplification of human and murine cDNAs using exon 5 and 6 primers. Total RNA extracted from normal human (top panel) or mouse (bottom panel) breast tissues (1–2), ovaries (3–4) and uteri (5–6) was reverse transcribed and PCR amplification was carried out using primers located in exons 5 and 6. The PCR product obtained in human tissues migrated at the size of 160 bp corresponding to ER-β wild-type (WT). PCR products obtained in mouse tissues migrated at the sizes of 177 and 123 bp, corresponding to a 54 bp inserted ER-β variant (54 bp Ins) and ER-β wild-type (WT), respectively.

A possible mechanism associated with the frequent inclusion of the inserted sequences in mouse ER-β transcripts may be the presence of several putative exonic splicing enhancer sequences within the insertion sequences (Cooper and Mattox, 1997; Coulter et al., 1997). Both purine rich motifs and A/C-rich splicing enhancer sequences are present (brackets in Fig. 4). The frequent inclusion of the inserted sequences in mouse ER-β transcripts, and their presence in several tissues at comparable levels with the wild-type transcript suggest that the protein encoded by the inserted transcript has a functional role, at least in the mouse and rat (Chu and Fuller, 1997). The putative function of a protein encoded by the inserted mouse ER-β transcript is unknown, and since the insertion is in the middle of the ligand binding domain, it may either disrupt binding completely or result in a different ligand binding specificity and/or affinity. Further, the insertion may effect the three dimensional structure of the ER-domain such that alterations in dimerization, transactivation and interaction with co-regulators may also occur. This could result in the inserted ER-β having a regulatory function on the wild-type ER-β as previously suggested (Chu and Fuller, 1997), or may completely alter its ability to heterodimerize and affect the activity of ER-α (Cowley et al., 1997). The lack of detection of a similar inserted ER-β transcript in human tissues may be due to hormonal differences at the time of tissue collection between the mouse and human subjects, or to a real species difference in alternative splicing. In the latter instance differences in alternative splicing between mouse and human with regard to a steroid hormone receptor have been previously documented (Oakley et al., 1996; Otto et al., 1997). Similarly, inserted sequences within the ligand binding domain of ER-α have also been reported (Murphy et al., 1996). In contrast with the inserted ER-β mRNA, the inserted ER-α mRNA was detected in one human breast tumor sample, and was due to a point mutation in one allele of the human ER-α gene present in the breast tumor (Wang et al., 1997).

In summary, in this report deletion splice variants of ER-β have been characterized for the first time in several mouse and human tissues, both normal and neoplastic. An inserted splice variant of the ER-β mRNA, previously identified in the rat, has been confirmed in several mouse tissues. This inserted variant was undetected in any human tissues analyzed, suggesting species specific differences in its expression.

Acknowledgements

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APPENDIX 7

1998, J. Clin. Endocrinol. Metab., In press

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ESTROGEN RECEPTOR BETA: MINE IS LONGER THAN YOURS ?

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Two years after the cloning of the second estrogen receptor, ER–β, the primary sequence encoding the N-terminus of the protein still remains uncertain. The demonstrated importance of estrogen signalling in normal and abnormal development of multiple tissues justifies characterization of this region.

Estrogens, involved in the normal development of a wide tissue spectrum including breast, uterus, brain and bone, are also implicated in several diseases such as breast and endometrial cancers, and osteoporosis. In 1995, the cloning (1) of ER–β from a rat prostate cDNA library, led to the need to fully re-evaluate estrogen signalling in target tissues. Like estrogen receptor alpha (2), rER–β is a ligand-dependent transcription factor which binds estrogen and antiestrogen. Relying on the presence of an in-frame stop codon upstream of their coding sequence, Kuiper et al. considered their open reading frame to encode the full length protein (R1, Fig.1). Shortly thereafter, a human homologue, hER–β, was cloned (3), from testis (H1, Fig.1). This cDNA encoded a protein 8 amino-acids shorter than rER–β. In 1997, the hER–β cDNA sequence was extended (4) to a start codon corresponding to the first rat methionine codon (H2, Fig.1), and the mouse homologue, mER–β, was cloned (5) (M1, Fig.1).

In 1998, following cDNA library screening and/or polymerase chain reaction, the N-terminus of ER–β was extended in all three species. Ogawa et al. (6) isolated from human testis a hER–β cDNA which could encode 45 additional amino-acids (H3, Fig.1). This sequence plus the presence of an upstream in-frame stop codon was recently confirmed by Moore et al. (7). In April 1998, a prostate rER–β cDNA sequence was submitted to Genbank, that differs from the initial sequence by only one base upstream of the start codon. This extra base alters frame and removes the previously observed in-frame stop codon, resulting in a cDNA which could encode 64 additional N-terminal amino-acids (R2, Fig.1). In May 1998, two sequences

submitted to Genbank extended the initial 5'-extremity of the mER–β cDNA. The encoded mER–β proteins contain 45 and 64 extra amino-acids N-terminal to M1 (M2 and M3, Fig.1), respectively. All three species present strong sequence similarities in this N-terminal region, although the rodent open reading frames are 19 amino-acids longer than the human.

Altogether, these observations raise important questions. Are cloning strategies and/or tissue/species specific expression of different forms of ER–β responsible for observed discrepancies? Do longer forms of ER–β, as yet unidentified, exist? If so, how is expression regulated? What are the functions associated with these extra amino-acids? The presence of putative phosphorylation and glycosylation motifs within these additional amino-acid sequences suggests possible regulation of putative function(s). Moreover, because of the demonstrated importance of the N-terminal region in ER–α, particularly involvment of AF-1 domain in hormone independent activation of the receptor (8), the elucidation of the function(s) associated with these new regions is necessary. The majority of functional studies undertaken to date were performed with constructs lacking these N-terminal amino-acids: addressing the above questions is critical to fully understand the involvement of ER–β in estrogen signalling.

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Figures legend

Figure 1

Schematic representation of rat, human and mouse ER–β protein. The amino-acid composition of N-terminal extremities of ER–β proteins predicted from cloned cDNAs is shown. For each species (rat R, human H, mouse M), initiating methionine codons are indicated in their chronological order of identification (1, 2, 3). Similarities between the three species are indicated by blue boxes. Sequences underlined by a single and double line represent putative phosphorylation and glycosylation motifs, respectively. Genbank accession numbers of sequences encoding R1, R2, H1, H3, M1, M2, and M3 are U57439, AJ002602, X99101, AB006590, U81451, AF063853, and AF067422, respectively.

A/B
C
D
E
F
DNA-binding
Ligand-binding
ER-β
R1
R2
H1
H2
H3
M1
M2
M3
rER-β
hER-β
mER-β

Figure 1

APPENDIX 8

1998, Cancer Res., 58, 3197.

[CANCER RESEARCH 58, 0000–0000, August 1, 1998]

Advances in Brief

Altered Estrogen Receptor α and β Messenger RNA Expression during Human Breast Tumorigenesis[1]

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Abstract

Using a multiplex reverse transcription-PCR assay, we compared the relative expression of estrogen receptor (ER) α and ER-β mRNA between adjacent samples of normal breast tissue and matched primary breast tumors obtained from 18 different patients. Within this cohort, 7 tumors were ER negative, and 11 tumors were ER positive, as determined by the ligand binding assay. No differences in the ratio of ER-α:ER-β expression were observed in the ER-negative cohort. However, in the ER-positive cohort, a significantly ($P < 0.02$) higher ER-α:ER-β ratio was observed in the tumor compared with that of the normal tissue component. Our data revealed that the increase in the ER-α:ER-β ratio was due primarily to a significant ($P < 0.05$) increase in ER-α mRNA expression in conjunction with a lower ER-β mRNA expression in the tumor compared with that of the normal compartment in some, but not all, ER-positive cases. These results suggest that the role of ER-α- and ER-β-driven pathways and/or their interaction change during breast tumorigenesis.

Introduction

Until recently, estrogen action was thought to be mediated principally through a single ER[3], ER-α, a member of the steroid/thyroid/retinoic acid receptor superfamily (1). As with other members of the family, the ER-α protein consists of several structural and functional domains (A–F). The NH_2-terminal transactivation function (AF-1) of the receptor is located within the A–B regions, whereas the DNA binding, the ligand-binding domain, and the second transactivation function (AF-2) reside in the C and E regions of the molecule, respectively (2). Upon ligand binding, conformational changes occur, and two ER-α molecules complexed with the hormone bind specifically to EREs located upstream of target genes. Interactions between ER-α and accessory proteins ultimately lead to the modification of the transcription of these genes (3). Similarly, the ER-α/estrogen complex can interact with c-*fos*/c-*jun* complexes to activate the transcription of target genes through activator protein 1 enhancer elements (4). Recently, a second ER, ER-β, was identified in the human, rat, and mouse (5–7). ER-β shares a similar structural and functional composition with ER-α and was also shown to activate the transcription of target genes through similar EREs (5, 8). However, differential activation of ER-α and ER-β by the antiestrogen 4-hydroxytamoxifen has been shown with ERE-regulated reporter genes (9), and the two ERs also show differential activation of activator protein 1-regulated reporter genes (10), suggesting different roles for these two receptors. In addition, because heterodimerization of ER-α and ER-β has been demonstrated *ex vivo*, putative cross-talk of the two signaling pathways has been suggested (11). The tissue-specific expression of ER-α and ER-β, although not identical, shows some overlap. It has therefore been speculated that estrogen action in a given tissue may depend on the relative expression of these two receptors.

The recent demonstration of ER-β expression in both human breast tumors (12–14) and normal human breast tissue (14, 15) suggests that the well-documented role of estrogen in breast tumorigenesis (16) may also involve both receptors. To determine whether altered expression of these two receptors might occur during breast tumorigenesis, we have compared the relative expression of ER-α and ER-β mRNAs in normal human breast tissues adjacent to matched primary breast tumors.

Materials and Methods

Human Breast Tissues and Cell Line. Eighteen cases were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). For each case, matched adjacent normal and tumor frozen tissue blocks were available. The quality of each block was determined by the histopathological assessment of sections from adjacent mirror image paraffin-embedded tissue blocks, as described previously (17). The presence of normal ducts and lobules (median $n = 6$; range, 2–13) as well as the absence of any atypical lesion was confirmed in all normal tissue specimens. Seven tumors were ER negative (ER < 3 fmol/mg protein), with progesterone receptor values ranging from 2.2–11.2 fmol/mg protein, as measured by the ligand binding assay. Eleven tumors were ER positive (range, 3.5–159 fmol/mg protein), with progesterone receptor values ranging from 5.8–134 fmol/mg protein. These tumors spanned a wide range of grade (grade, 5–9), which was determined using the Nottingham grading system. For all normal and tumor samples, the percentage of epithelial cells, stromal component, and fat has been estimated by observation of the adjacent paraffin-embedded sections. For normal tissue, the median of the percentage of epithelial cells, stroma, and fat observed within the sections was 10 (range, 5–30%), 50 (range, 5–85%), and 40% (range, 5–90%), respectively. For tumor tissues, the median of the percentage of epithelial tumor cells, normal epithelial cells, stroma, and fat within the sections was 40 (range, 20–60%), 2.5 (range, 0–10%), 37.5 (range, 20–65%), and 20% (range, 10–50%), respectively. Three tumors (T1, T2, and T3) shown in a previous study (12) to express low ER-β/high ER-α, high ER-β/low ER-α, and high ER-β/high ER-α mRNA levels, respectively, were used to validate a multiplex RT-PCR that was designed to determine the relative expression of ER-α:ER-β mRNA. MDA-MB-231 cells were grown and harvested, and the cell pellets were stored at −70°C, as described previously (12). Total RNA was extracted from 20 μm of frozen tissue sections (15- and 5-μm sections for normal and tumor breast tissue, respectively) or cell pellets using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. One μg of total RNA was reverse-transcribed in a final volume of 25 μl as described previously (12).

Primers and PCR Conditions. The primers used consisted of ER-β-U primer [5′-GTCCATCGCCAGTTATCACATC-3′ (sense), located in ER-β 130–151] and ER-β-L primer [5′-GCCTTACATCCTTCACACGA-3′ (anti-

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[3] The abbreviations used are: ER, estrogen receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ERE, estrogen-responsive elements.

sense), located in ER-β 371–352]. The nucleotide positions given correspond to published sequences of human ER-β cDNA (5). The other pair of primers used consisted of ER-α-U primer [5′-TGTGCAATGACTATGCTTCA-3′ (sense), located in ER-α 792–811] and ER-α-L primer [5′-GCTCTTCCTC-CTGTTTTTA-3′ (antisense), located in ER-α 940–922]. The nucleotide positions given correspond to published sequences of human ER-α cDNA (1). PCR amplifications were performed, and PCR products were analyzed as described previously, with minor modifications (12). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl in the presence of 1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 2 ng/μl ER-α-U/ER-α-L and/or 4 ng/μl ER-β-U/ER-β-L, and 0.3 unit of Taq DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. After electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed *GAPDH* cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (12). PCR products were subcloned and sequenced as described previously (12).

Multiplex PCR Validation. Total RNA was extracted from MDA-MB-231 cells previously shown to express very low ER-α but higher ER-β mRNA levels (12) and from tumors T1, T2, and T3, the characteristics of which are described above. In the first series of experiments, six cDNA preparations were prepared that contained varying percentages of MDA-MB-231 and tumor T1 cDNA by mixing 10, 8, 6, 4, and 0 μl of MDA-MB-231 cDNA with 0, 2, 4, 6, 8, and 10 μl of tumor T1 cDNA (0, 20, 40, 60, 80, and 100% T1 cDNA, respectively). The same experiment was reproduced using a 10-fold dilution of these six cDNA preparations. A second series of experiments was performed in which the six cDNA preparations contained a constant amount of MDA-MB-231 cDNA (5 μl) and 0, 1, 2, 3, 4, and 5 μl of T1 cDNA in a final volume of 10 μl (0, 10, 20, 30, 40, and 50% T1 cDNA, respectively). A third series of experiments contained six cDNA preparations in which the amount of T1 cDNA was held constant (5 μl) with increasing amounts of 0, 1, 2, 3, 4, and 5 μl of MDA-MB-231 cDNA in a final volume of 10 μl (0, 10, 20, 30, 40, and 50% MDA-MB-231 cDNA, respectively). Finally, 1 μl of T1, T2, and T3 cDNA was amplified independently for 22, 26, 30, and 34 cycles. In every case, PCR products were separated on 6% polyacrylamide gels containing 7 M urea. After electrophoresis, the gels were dried and autoradiographed. Signals were quantified by excision of the appropriate bands, the addition of 5 ml of scintillant (ICN Pharmaceuticals, Inc., Irvine, CA), and counting in a scintillation counter (Beckman Instruments).

Quantification and Statistical Analyses. To quantitate ER-α mRNA expression relative to ER-β mRNA expression, coamplification of ER-α and ER-β cDNAs was performed using the multiplex PCR described above. Quantification of the signals was carried out by the excision of the bands corresponding to ER-α and ER-β cDNAs, the addition of scintillant, and scintillation counting. Three independent PCRs were performed. To control for variations between experiments, a value of 100% was assigned to the highest signal measured in each set of PCR experiments, and all signals were expressed as a percentage of this signal. Indeed, the same tissue sample showed the highest signal in all experiments. For each sample, the ER-α:ER-β ratio was calculated. Differences between averages of log(ER-α:ER-β) obtained for matched normal and tumor compartments were tested using the two-tailed Wilcoxon signed rank test.

Evaluation of ER-α and ER-β mRNA expression was performed by independent amplification of both ER-α and ER-β cDNA, *i.e.* using ER-α- or ER-β-specific primers. Two independent PCRs were performed. To control for variations between experiments, all signals were expressed as a percentage of the highest signal observed. In parallel, *GAPDH* cDNA was amplified, and after analysis of the PCR products on prestained agarose gels, the signals were quantified by scanning using NIH Image 161/ppc software. Each *GAPDH* signal was also expressed as a percentage of the highest signal observed in the experiment. The average of ER-α and ER-β signals was then expressed as a percentage of the *GAPDH* signal. Differences between matched normal and tumor elements were tested using the two-tailed Wilcoxon signed rank test. Correlations were tested by calculation of the Spearman coefficient (*r*).

Results

Multiplex PCR as an Approach to Determine the Relative Expression of ER-α and ER-β. To determine the relative expression of ER-α and ER-β mRNA within any individual sample, we used a multiplex PCR assay. In this assay, two set of primers are added to each individual PCR, thus allowing the coamplification of both ER-α and ER-β cDNA in a single tube and therefore eliminating variation introduced due to differences in cDNA loading. To determine whether the results obtained from the multiplex PCR assay directly reflected the initial ER-α:ER-β cDNA ratio, a series of preliminary experiments was conducted. In these experiments, four different cDNA preparations were used. MDA-MB-231 cells, breast tumor T1, breast tumor T2, and breast tumor T3 had been previously shown to contain high ER-β/low ER-α, very low ER-β/high ER-α, high ER-β/low ER-α, and high ER-β/high ER-α mRNA levels, respectively (12). The first experiment consisted of the multiplex amplification of spiked cDNA preparations containing various percentages of MDA-MB-231 and T1 cDNAs (Fig. 1). As shown in Fig. 1*A*, the PCR signal corresponding to ER-β in MDA-MB-231 cells decreased with decreasing input of MDA-MB-231 cDNA, and the ER-α signal increased with increasing input of T1 cDNA. Quantification of the ER-α:ER-β ratio signals was plotted as a function of the percentage of T1 cDNA input (Fig. 1*B*). A direct relationship was found. Similar results were obtained using a 10-fold dilution of the cDNA preparations (data not shown). Using a constant amount of MDA-MB-231 cDNA plus or minus increasing amounts of T1 cDNA (containing primarily ER-α cDNA), a linear increase in the ER-α:ER-β ratio with increasing ER-α input (T1 cDNA) was found (Fig. 2, *A* and *B*). An inverse but linear relationship was obtained using a constant amount of T1 cDNA and increasing amounts of MDA-MB-231 cDNA input (data not shown). Finally, the rank of ER-α:ER-β ratios in T1, T2, and

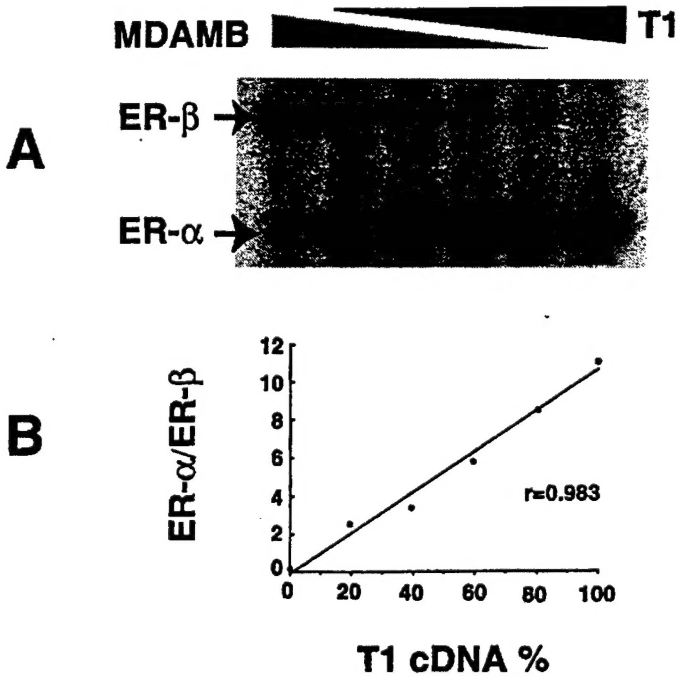

Fig. 1. Multiplex amplification of MDA-MB-231 (low ER-α/high ER-β content) and tumor T1 (low ER-β/high ER-α content) cDNA mixed preparations. An aliquot of solutions containing an increasing amount of tumor T1 cDNA and a decreasing amount of MDA-MB-231 cDNA was prepared and amplified by PCR using ER-α- and ER-β-specific primers in a single tube, and PCR products were separated on an acrylamide gel as specified in "Materials and Methods." *A*, autoradiograph of the gel. *B*, the ER-α:ER-β ratio is expressed as a function of the percentage of tumor T1 cDNA contained in the initial preparation.

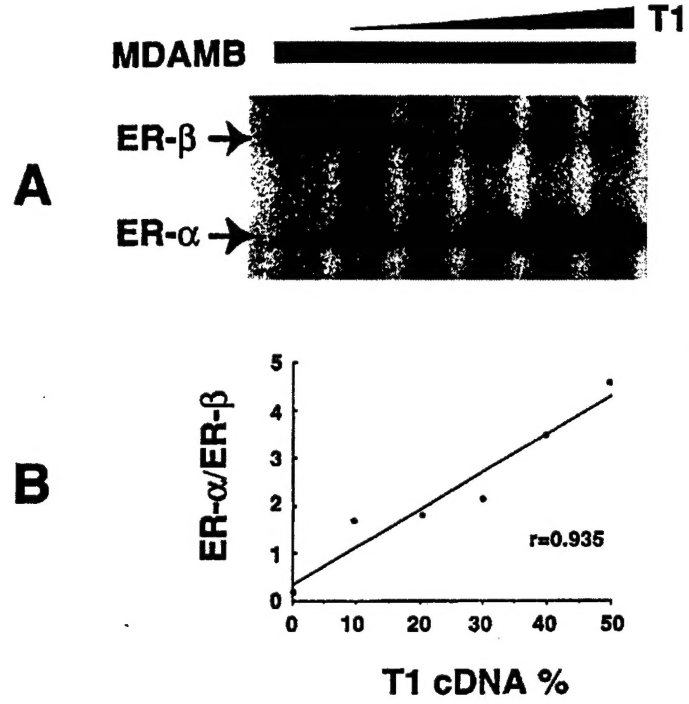

Fig. 2. Multiplex amplification of a constant amount of MDA-MB-231 (low ER-α/high ER-β content) cDNA and an increasing amount of tumor T1 (low ER-β/high ER-α content) cDNA. An aliquot of solutions containing a constant amount of MDA-MB-231 cDNA and an increasing amount of tumor T1 cDNA was prepared, amplified by multiplex PCR, and separated on an acrylamide gel as specified in "Materials and Methods." *A*, autoradiograph of the gel. *B*, the ER-α:ER-β ratio signals is expressed as a function of the percentage of tumor T1 cDNA contained in the initial preparation.

T3 using the multiplex PCR assay was not affected by the number of cycles used for the PCR over a range of 22–34 cycles. The ranking was similar to that deduced using several independent PCR determinations of the ER-α and ER-β mRNA levels, *i.e.* T1 ER-α:ER-β > T3 ER-α:ER-β > T2 ER-α:ER-β (data not shown). Multiplex PCR performed under the described conditions therefore seemed to be a reliable method with which to compare small tissue samples for their relative expression of ER-α and ER-β mRNA.

Comparison of the Relative Expression of ER-α:ER-β mRNA in Adjacent Normal Breast Tissue and Matched Primary Breast Tumors. To determine whether alterations may occur in the contribution of ER-α and ER-β signaling during breast tumorigenesis, the relative expression of ER-α and ER-β mRNA was measured in matched normal and primary tumor tissues from 18 different patients. Within the cohort of tumors studied, 7 tumors were ER negative, and 11 tumors were ER positive, as determined by the ligand binding assay. Total RNA was extracted from the frozen tissue sections and analyzed by multiplex RT-PCR. Examples of the results obtained are shown in Fig. 3*A*. In both normal and tumor compartments, two PCR products migrating at an apparent size of 242 and 149 bp were obtained. These PCR products were identified by cloning and sequencing to correspond to ER-β and ER-α cDNA, respectively. ER-α and ER-β signals obtained in three independent multiplex PCRs were quantified as described in "Materials and Methods." The ER-α:ER-β ratio was calculated for each sample, and the results for each matched sample are presented in Fig. 4*A*. Considering all cases together, no significant change in the ER-α:ER-β ratio was observed between normal and tumor compartments. The cases were then divided into two groups based on the ER positivity of their tumors. Once again,

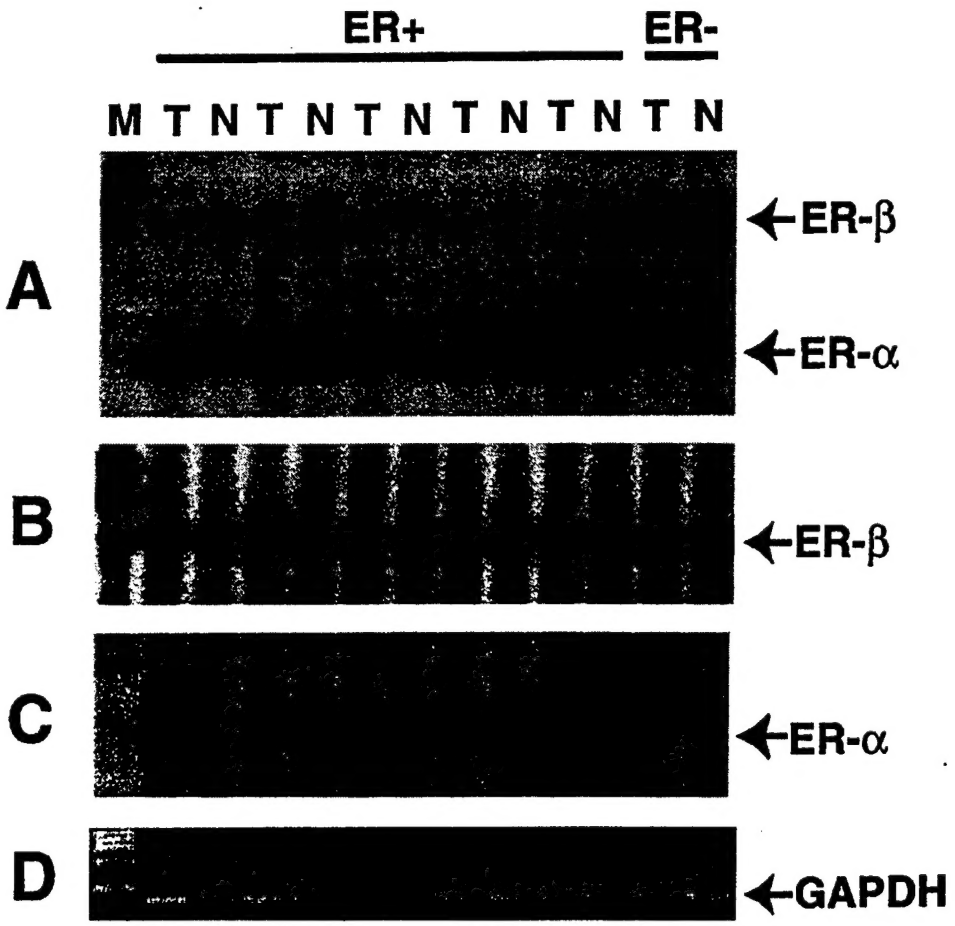

Fig. 3. RT-PCR analysis of ER-α and ER-β expression within matched normal and tumor compartments of human breast tumors. Total RNA was extracted from matched normal (*N*) and tumor (*T*) compartments of ER-positive (*ER+*) or ER-negative (*ER−*) tumors, as determined by ligand binding assay. Corresponding cDNA was amplified, and PCR products were separated on an acrylamide gel, as described in "Materials and Methods." PCR was performed using: *A*, ER-α and ER-β primer sets in a single tube (multiplex PCR); *B*, ER-β-specific primers only; *C*, ER-α-specific primers only. *D*, for each sample, *GAPDH* cDNA was amplified in parallel, and PCR products were separated on an agarose gel, as described in "Materials and Methods." *M*, molecular weight marker (φx174 RF DNA/*Hae*III fragments; Life Technologies, Inc.).

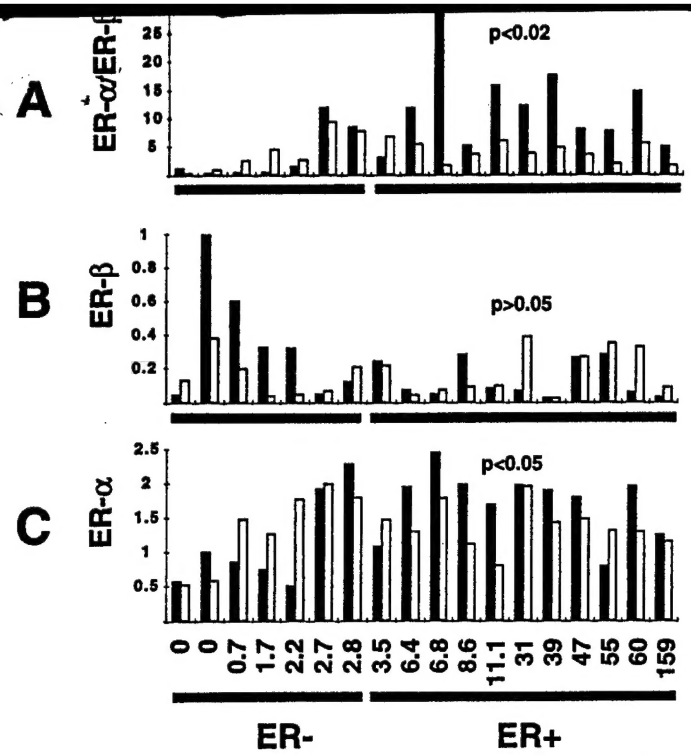

Fig. 4. Quantification of ER-α and ER-β expression within matched normal and tumor compartments of human breast tumors. Total RNA extracted from matched normal (□) and tumor (■) compartments of ER-positive (*ER+*) or ER-negative (*ER−*) tumors was reverse-transcribed and PCR-amplified, and PCR products were separated on an acrylamide gel, as described in "Materials and Methods." Signals have been quantified and normalized, as indicated in "Materials and Methods." *A*, ER-α:ER-β ratio obtained after multiplex PCR. *B*, ER-β signals obtained after PCR was performed using ER-β-specific primers only. *C*, ER-α signals obtained after PCR was performed using ER-α-specific primers only. ER status of the tumor component, as assessed by ligand binding assay, is indicated (fmol/mg protein). Differences between matched normal and tumor compartments in ER-positive cases were tested using the two-tailed Wilcoxon signed rank test.

within the ER-negative cohort, no difference in the ER-α:ER-β ratio was seen between normal tissue and matched tumors. In contrast, in the ER-positive tumor group, a significant increase (two-tailed Wilcoxon signed rank test, $P < 0.02$) in the ER-α:ER-β ratio was observed in the tumor compartment compared with that of the normal compartment. It should be stressed that a significant correlation was found between the ER-α:ER-β ratio observed in the tumor compartment and ER status by binding (Spearman $r = 0.603$; $P < 0.01$).

Independent Measurement of ER-α and ER-β mRNA Expression within Matched Normal and Tumor Compartments. The observed increase in the ER-α:ER-β mRNA ratio of ER-positive breast tumors *versus* matched normal tissue could result from a decrease in the absolute levels of ER-β mRNA and/or an increase in the absolute ER-α mRNA levels within the tumor compartment relative to the matched normal tissue. To distinguish between these possibilities, the ER-β and ER-α mRNA levels were determined individually in each sample by RT-PCR, using either ER-β-specific primers or ER-α-specific primers. Examples of the results obtained are shown in Fig. 3, *B* and *C*, respectively. In parallel, amplification of the ubiquitously expressed *GAPDH* cDNA was also performed (Fig. 3*D*). For each sample, the ER-β and ER-α cDNA signals were quantified, and the average of signals obtained in two independent PCRs was normalized to the *GAPDH* signal, as described in "Materials and Methods." The results are shown in Fig. 4, *B* and *C*, for ER-β and ER-α, respectively. No significant change in ER-β or ER-α mRNA expression was observed between the normal and tumor compartments within the ER-negative cases. Although the difference was higher in the normal compartment *versus* the matched tumor component in 8 of 11 (72%) ER-positive cases. A significant (two-tailed Wilcoxon-signed rank test, $P < 0.05$) increase in ER-α mRNA expression was measured in the tumor compartment of ER-positive tumors compared with that of the matched normal tissues. ER-α and ER-β signals observed in normal or tumor compartments did not correlate with the cellular composition of the section analyzed, *i.e.* percentage of normal epithelial cells, tumor epithelial cells, stroma, or fat (data not shown). One should note that although not statistically significant, trends toward an association between ER status by binding and ER-α (Spearman $r = 0.397$; $P = 0.10$) and ER-β (Spearman $r = -0.4254$; $P = 0.07$) have been observed.

Discussion

The discovery of the expression of a second ER in both normal and neoplastic human mammary tissues (12–15), together with the known perturbations of estrogen and antiestrogen sensitivity during breast tumorigenesis and breast cancer progression (16, 18–21), necessitates an investigation of the function of ER-β in human mammary tissue and a reevaluation of the estrogen signal transduction system in these tissues. We have used a multiplex assay in which ER-α and ER-β cDNA are amplified in the same reaction to investigate the relative expression of ER-α and ER-β mRNAs between adjacent samples of normal breast tissue and matched primary breast tumors. The choice of an RT-PCR-based approach to address the question of the relative expression of both receptors has been dictated by several parameters: (*a*) the absence of any publication to date using antibodies to detect ER-β protein in human breast tissue suggests that reliable antibodies are not yet available for this purpose; and (*b*) the expression of ER-β mRNA is relatively low in breast tissue, as demonstrated by the time needed to observe a signal in epithelial human breast cells in *in situ* hybridization studies (14) and by much weaker signals obtained, compared with ER-α, when analyzing breast tissue samples by RNase protection assay.[4] The multiplex PCR assay developed here seems to be a reliable method with which to compare tissue samples for their relative expression of ER-α and ER-β mRNA. It should be stressed, however, that despite the good overall correlation coefficient observed, samples with an ER-α:ER-β ratio of <2 may be less reliably compared with each other (Fig. 2). This could possibly be a limitation of the multiplex approach, which would likely have a higher impact when comparing ER-negative tumors, in which ER-α is known to be weakly expressed. Such a limitation of multiplex PCR analysis of genes expressed at very low levels has previously been reported and may be circumvented by increasing cDNA input (22).

Our data show that in the cohort of patients whose tumors are ER positive by ligand binding, the ratio of ER-α:ER-β is significantly higher in breast tumors than it is in adjacent normal tissues. This difference seems mainly due to an up-regulation of ER-α mRNA levels within the tumor compartment. This observation is in agreement with previous published data showing a generally higher expression of ER-α detected immunohistochemically in ER-positive breast tumors than in normal breast tissue (see Ref. 23 and references herein). However, it is possible that down-regulation of ER-β expression in the tumor tissue may also contribute to the altered ratio in some tumors. Although the difference did not reach statistical significance, 72% of the ER-positive cohort showed a trend in which ER-β expression was lower in the tumor when compared with the normal compartment. The study of larger numbers of cases will be necessary to confirm this trend. Similarly, although no correlations have been

[4] Unpublished observation.

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observed between the expression of ER-α and ER-β assessed by multiplex PCR and the cellular composition of the sections analyzed, one cannot exclude the possibility that such relationships might exist. The study of a larger number of samples will also clarify this issue. If these RNA studies are paralleled at the protein level, then our data suggest that a significant change in the ratio of these two ERs occurs between normal and neoplastic breast tissues. This would further suggest that the contribution of ER-α- and ER-β-driven pathways and/or their interactions changes in conjunction with breast tumorigenesis. The hypothesis that such changes in ER-α and ER-β signaling pathways may occur during tumorigenesis is also supported by the recent observations of Brandenberger *et al.* (24). These authors showed that ER-α mRNA expression is equal or slightly higher in ovarian cancer tissues compared with normal ovary tissues, and ER-β mRNA expression is decreased in ovarian tumor tissue. The measurement of the ER-α:ER-β ratio correlated with ER status as assessed by ligand binding assay. Moreover, trends toward a positive correlation between ER-α and ER status and toward a negative correlation between ER-β and ER status were observed. Together, these data suggest that ligand binding is mainly due to the ER-α protein.

We have previously observed that the apparent ER-α:ER-β ratio in breast tumors varies widely (12). Our current results using the multiplex RT-PCR approach confirm and support these previous observations. Given the differential activity of tamoxifen-like antiestrogens through ER-α and ER-β, it is tempting to speculate that altered ratios of these receptors may be a possible mechanism associated with tamoxifen resistance.

In conclusion, our results provide evidence to support the hypothesis that altered ER-α and ER-β expression may have a significant role in alterations of estrogen action that occur during human breast cancer.

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change page numbers: 38-44